Bile Lipid Secretion in Isolated Perfused Rat Liver. A Model for Metabolic Studies

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Isolated perfused rat liver was used to study the effects of constant taurocholate perfusion, with or without the addition of phosphatidylcholine unilamellar vesicles, upon both the bile salt-dependent and bile salt-independent secretion of bile. Taurocholate introduction increased bile flow and normalized the bile lipid secretion by restoring the bile salt-dependent secretion. At a flow rate of 30 ml/min, the liver was perfused by a single-pass method. The perfusion medium contained 17.5 μM taurocholate with or without 5.83 μM phosphatidylcholine. In light of a recent quantitative dynamic concept on the interphase partition of lipids, it was calculated that more than 99% of the taurocholate reaches the liver as monomers and/or dimers. It was also deduced that the lipids were secreted in bile as small discoidal lipoprotein structures rather than unilamellar lipoproteic vesicles. During the course of the experiments (2 hr), the excellent criteria of viability of this model make it highly suitable for the investigation of hepatic metabolism. Furthermore, the addition of phosphatidylcholine unilamellar vesicles to the perfusate constitutes a potential vector for various liposoluble molecular species.
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Isolated perfused rat liver is a valuable model for the investigation of physiological, biochemical and pharmacological aspects of liver functions and has been widely used for this purpose by many investigators (1,2).

Under physiological conditions, the rate of bile lipid secretion is dependent on the amount of bile salts (BS) returned in the portal blood (3). Most bile salts stored in the intestinal tract reach the liver in this way (4,5), since the portal flow is 500 times higher than the intestinal lymph flow (6). Preservation of a physiological enterohepatic circulation of BS is a prerequisite for normal bile lipid secretion. Interruption of this circulation by biliary diversion induces a progressive decrease in the bile lipid secretion correlated with the disappearance of the intestinal BS pool. This disappearance is not compensated by a sufficient increase in BS synthesis in the liver. In isolated perfused liver without perfused BS, this phenomenon is rapidly and dramatically accentuated since the liver pool of BS is very small compared to the BS pool in the intact enterohepatic circulation.

The BS-independent secretion of bile is correlated with secretion of water and electrolytes (7,8). BS-dependent bile secretion is an active hepatic process indispensable for normal bile secretion of lipids (9,10), i.e., mainly phosphatidylcholine (PC) and free cholesterol. Most authors have assumed that bile lipids are mixed micelles or unilamellar vesicles (11,12). However, our results (13-15) show that bile lipids may occur as a bile lipoprotein complex (BLC), i.e., that micellar-like structures or unilamellar vesicles are associated electrostatically with IgA fragments and hydrophobically with an anionic polypeptide fraction (APF), which is different from the well-known plasma apolipoproteins (14) that are detected in trace amount in bile by radioimmunoassay (16). Bile contains small amount of plasma, and immunoelectrophoresis of concentrated bile (obtained after delipidation and lyophilization) gives a protein pattern similar to plasma (17), thus explaining the presence of apolipoproteins. Due to their own osmotic activity, BS molecularly dispersed in the aqueous polar phase actively taken up by hepatocytes in the sinusoidal pole (space of Disse) display a choleretic effect. Isolated perfused rat liver is a widely used model for the study of drug biotransformations and biliary excretion of their metabolites. Up to now, however, most of the isolated perfused liver models have taken into account only BS-independent bile secretion and neglected BS-dependent bile secretion (18-20). A recent exception to this oversight is the data of Lowe et al. (21), showing the effect of an intraportal BS bolus on the secretion of PC and free cholesterol. The purpose of the present study was to ascertain the effect of a constant BS perfusion. Under this condition, a stable biliary secretion of both the BS-dependent and BS-independent fractions was observed.

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MATERIALS AND METHODS

Liver perfusion. To minimize anoxia of the liver, the portal vein was rapidly catheterized (30 sec). Krebs-Henseleit buffer (22), pH 7.3, containing 2 mM of calcium chloride, 8 mM of glucose and 2% Dextran was infused at a constant flow rate of 30 ml/min. The perfusate was maintained at 38 ± 0.5 C and continuously oxygenated with an O_2:CO_2 (95:5, v/v) mixture at a flow rate of 2 l/min. Osmolarity was monitored with the Fiske 330 D osmometer (the values found were 300 ± 5 mOsm/l). After this procedure, the bile duct was catheterized. The whole operating procedure lasted ca. 10 min. The equipment used was based on the model proposed by Brunengraber et al. (23) under nonrecirculating conditions. The liver left in situ was kept moist throughout the experiments.

Test animals were divided into three groups, one control and two experimentals. The control group (group 1) had two animals and was perfused with buffer without taurocholate (TC) and PC. The two experimental groups (five rats per group) were perfused either with buffer containing 17.5 μM of TC only (group 2) or with buffer containing 17.5 μM of TC and 5.83 μM of PC (group 3). In the experimental groups, after stabilizing the system for 15 min, ^{14}C-TC (1.28 μCi) in 20 ml of perfusion medium was also administered at a flow rate of 1 ml/min. Bile sampling was performed every 20 min in preweighed tubes. The volume of secretion was recorded in grams (bile density equal to 1 g/ml). Alanine amino transferase (ALAT) and aspartate amino transferase (ASAT) transaminase activity as well as creatinin phosphokinase (CPK) activity in the outflowing perfusate were checked every

10 min. The amount of radioactivity not bound by the liver was determined by sampling outflowing perfusate every 10 sec during ^{14}C-TC perfusion.

Chemicals. The following high grade chemicals were used: taurocholate A grade from Calbiochem Boehring Corp. (Meudon, France; purity by TLC 98%); phosphatidylcholine from Sigma (Paris, France; type III-E from frozen egg yolk in hexane, purity 99%); ^{14}C-TC in aqueous solution containing 2% ethanol from Amersham (Les Ullis, France; sp act 64 mCi/mmol); ALAT, ASAT and β-glucuronidase from Boehringer Mannheim Gmbh kit (Meylan, France); goat anti-rabbit IgG and alkaline phosphatase from Sigma; p-nitrophenol from Flow Lab. (Paris, France); NaCl, KCl, KH_2PO_4, $CaCl_2$, $MgSO_4$ and glucose from Farmiglia Carlo Erba (Paris, France); $NaHCO_3$ from Prolabo (Paris, France), and Dextran (mol wt 70,000) from Fluka (Buchs, Switzerland).

Dilution of mixed micelles in the perfusate. Mixed micelles were prepared by carefully evaporating to dryness 17.86 mg of PC in hexane solution under nitrogen and solubilizing the resulting residue in 10 ml of a micellar solution containing 37.60 mg of TC. As calculated in $mol \cdot cm^{-3}$ according to the quantitative dynamic concept on the interphase partition of lipids applied to TC-PC-water ternary system (13,24), the concentration of TC and PC, incorporated after the dilution into unilamellar vesicles, was $0.05 \cdot 10^{-4}$ $mol \cdot cm^{-3}$ and $12.73 \cdot 10^{-4}$ $mol \cdot cm^{-3}$, respectively, in the mixed bilayer interfacial lipid phase. Thus, since the PC/TC molar ratio in the vesicles averages 255, these vesicles can be considered pure PC unilamellar vesicles. On the other hand, the concentration of TC molecularly solubilized in the water polar phase is $17.48 \cdot 10^{-9}$ $mol \cdot cm^{-3}$, which is almost identical to the $17.50 \cdot 10^{-9}$ $mol \cdot cm^{-3}$ obtained with TC perfusate without PC. The advantage of adding TC-PC to the perfusate is that PC unilamellar vesicles can solubilize class 3 and 4 lipids (13,24) without appreciably affecting the concentration of TC molecularly solubilized in the water phase as monomers and dimers. The total inflow of PC through the liver (21 μmol) corresponds to ca. 8% of the total PC pool in the liver (averaging 260 μmol), while the amount of PC secreted in bile (5.1 μmol) corresponds to 2% of the PC content of the liver.

Analytical methods. PC were assayed by the semiautomatic method of Amic et al. (25), TC by the automatic enzymatic method of Domingo et al. (26), β-glucuronidase activity by the method of Fishman et al. (27) with a Sigma Kit and ALAT, ASAT, CPK activity in the perfusate by the Boehringer enzymatic method. ^{14}C-TC was assayed with a liquid scintillation spectrophotometer (Beckman LS 2800) in 5 ml of PCS (Amersham) scintillation fluid.

Detection of the anionic polypeptide fraction (APF) in rat bile. Anti-human APF specific polyclonal serum was prepared from rabbits by the procedure of Henry et al. (28). Work in progress using the ELISA method shows the presence of a common antigenic determinant in the amino acid sequence in human, rat, dog, calf and pig APF. The APF injected into the rabbits was purified using a procedure of zonal ultracentrifugation in 1–15% sucrose density gradient containing glycodesoxycholate at a concentration of 1.5 mM (15).

The principle of the ELISA assay (29,30) is the following: the wells in a polystyrene plate (LINBRO E.I.A. microtitration plate, Flow Laboratories) are coated with diluted rat bile (at a dilution of 1/200), or purified APF (0.5 to 20 μg per 100 μl according to automatic amino acid analysis) for the standard curve, in a carbonate buffer 0.1 M at pH 9.6, and then washed three times to remove the unbound components. The diluted polyclonal anti-APF serum (at a dilution of 1/200 in isotonic phosphate buffered saline, PBF) was added during 90 min at 37 C. The complex antigen-antibodies were washed three times. Bound antibodies are then detected using an anti-rabbit IgG serum (at a dilution of 1/200) conjugated to alkaline phosphatase (Sigma), and the activity was detected by the p-nitrophenylphosphate as the substrate (1 mg/ml). The enzymatic reaction was allowed to proceed for 30 min in the dark at 37 C. The absorbance was measured at 405 nm with a Multiskan Titertek MC (Flow Laboratories) against a blank.

Statistical method. Statistical significance of results was determined by the Student t-test.

RESULTS

Uptake of ^{14}C-TC by the liver is almost complete, and the radioactivity of the exiting perfusate is negligible. Its concentration in bile peaks 20 min after injection and then drops quickly during the following 60 min, regardless of whether the nonlabeled TC perfusion contains PC unilamellar vesicles (Fig. 1). The hepatic clearance of ^{14}C-TC is very high—95% (group 2, TC alone) and 98% (group 3, TC + PC vesicles). No significant difference was observed between these two groups.

Figure 2 shows the concentration of PC and BS recorded in the bile collected from the three groups. When TC with or without PC vesicles is present in the perfusate, PC and BS concentrations and BS/PC molar ratio (10.4 ± 0.3) remain within the physiological range. No significant

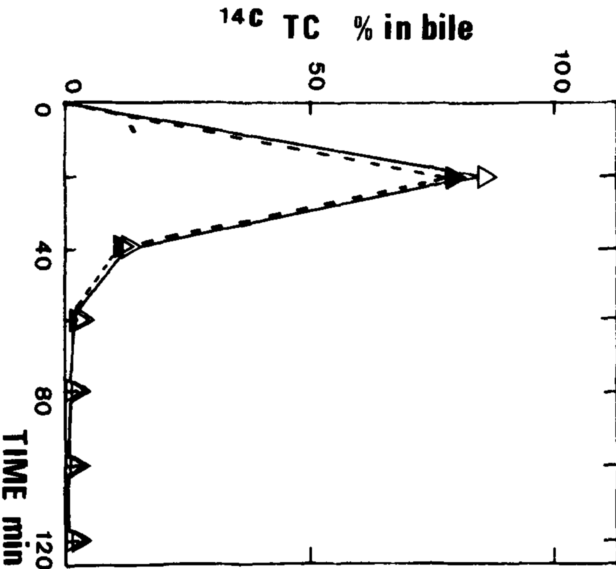
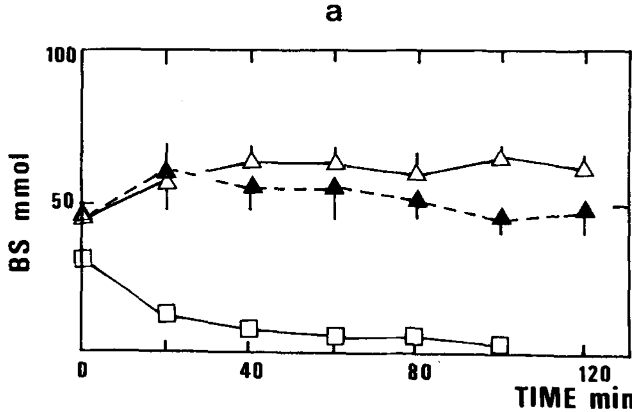
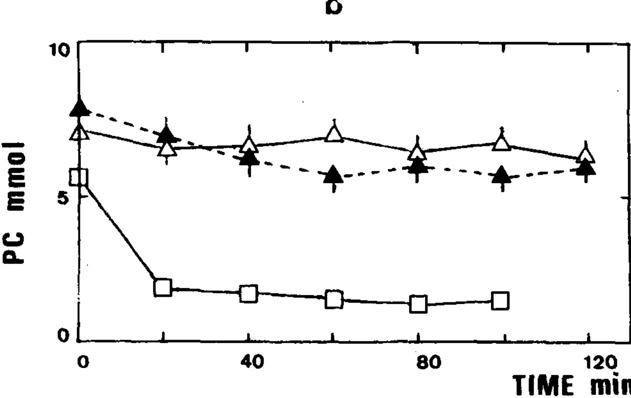


FIG. 1. Bile secretion of ^{14}C-bile salts in function of time in min from perfusion medium containing ^{14}C-taurocholate (TC) or ^{14}C-TC + PC unilamellar vesicles by isolated rat liver (see text). Each point represents the mean value ± SEM (n = 5). Group 2 (TC), △—△; group 3 (TC + PC), ▲—▲.





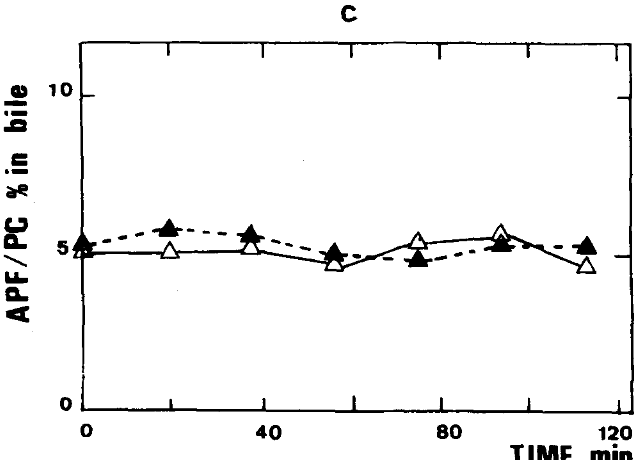


FIG. 2. Concentration of lipids in bile in function of time in min. (a) Bile salts (BS); (b) phosphatidylcholine (PC). Each point represents the mean value ± SEM (n = 5). (c) Determination of the APF/PC weight percentage (see text) in one rat of group 2 and one rat of group 3. Control, □—□; group 2 (TC), △—△; group 3 (TC + PC), ▲—▲.

difference was noted between groups 2 and 3. In group 1 (control), the concentration of PC and BS is very low, but the molar ratio PC/BS remains normal for 40 min. The APF/PC weight ratio in one rat of group 2 and one rat of group 3 (Fig. 2) remains stable (0.045–0.050).

As shown in Table 1, the bile flow from liver perfused with TC with or without PC unilamellar vesicles is 1.5 to 2 times higher than in livers perfused without TC. In experimental groups 2 and 3, bile flow remains stable throughout the 120-min test period.

TABLE 1

Biliary Flow (μl/min)

Time of collection (min)	Control (n = 2)	TC (n = 5)	TC + PC vesicles (n = 5)
0–20	5.5 ± 1.5	11.6 ± 1.68	9.8 ± 1.70
20–40	5.5 ± 1.5	10.8 ± 2.01	8.0 ± 2.37
40–60	5.0 ± 1.0	8.6 ± 2.57	9.1 ± 1.47
60–80	4.0 ± 1.0	8.4 ± 2.60	9.5 ± 1.44
80–100	4.5 ± 1.5	10.1 ± 1.37	9.2 ± 1.11
100–120	4.75 ± 1.75	10.1 ± 0.88	8.7 ± 0.91

Each value represents mean ± SEM in the three groups of rat.

TABLE 2

β-Glucuronidase Activity: Percent of Variation to Basal Value (0–20 min)

	TC	TC + PC vesicles
20–40 min (n = 3)	100	100
60–80 min (n = 3)	175.2 ± 55.0	190.8 ± 46.9
100–120 min (n = 3)	195.5 ± 35.7	145.4 ± 18.0

Although β-glucuronidase activity did double during perfusion (Table 2), it never exceeded the values previously reported in isolated perfused liver (31), but was nevertheless five times lower than physiological values in intact animals (32,33).

To check the viability of the liver, the ALAT, ASAT and CPK activities were assayed in the exiting perfusate. These activities were found to be normal in all three groups (Table 3).

DISCUSSION

The greatest advantage of our experimental model is that bile secretion is maintained at normal physiological levels. Also, perfusion with TC with or without PC unilamellar vesicles stabilized the concentration of BS and PC as well as the molar ratio BS/PC in bile (10.4 ± 0.3) during the 2-hr sampling period. The APF/PC weight ratio in bile studied in two rats remained constant, suggesting a normal formation of the bile lipoprotein complex. The absence of opalescence and the respective concentration of BS and PC in bile favor the presence of the bile lipoprotein complex as small discoidal lipoproteic structures rather than vesicles (13,20); it was previously observed (unpublished data) that, unlike human hepatic bile, the bile lipoproteic complex is present in rat bile only as the low mol wt pseudomicellar family, and not as large discoidal structures or lipoproteic vesicles (the rat is devoid of gallbladder with its concentrating activity). In control liver perfused without TC, lipid levels decreased rapidly and dramatically during the first 40 min after isolation due to an uncompensated wash-out of BS. Interestingly, the low secretion rate continued to be observed in controls; this may indicate the occurrence of

TABLE 3

Enzyme Activity in the Perfusion Medium[a]

Time of perfusion (min)	ALAT (UI/l)	ASAT (UI/l)	CPK (UI/l)
0	2.0 ± 0.46	2.75 ± 0.45	3.37 ± 0.86
10	3.12 ± 0.61	2.37 ± 0.46	1.62 ± 0.37
20	4.25 ± 0.61	2.50 ± 0.56	1.87 ± 0.44
30	5.25 ± 1.08	2.62 ± 0.98	2.37 ± 0.56
40	6.50 ± 1.75	4.37 ± 1.48	2.50 ± 0.42
50	9.37 ± 2.07	6.87 ± 2.29	3.25 ± 0.41
60	11.12 ± 2.59	9.25 ± 2.56	3.25 ± 0.41
70	13.87 ± 2.26	11.37 ± 2.77	5.50 ± 0.65
80	16.62 ± 2.54	14.12 ± 2.87	6.87 ± 1.0
90	18.25 ± 3.42	13.88 ± 3.92	6.37 ± 1.03
100	20.50 ± 2.78	18.50 ± 2.89	7.12 ± 0.95
110	23.25 ± 1.97	23.25 ± 3.25	10.12 ± 2.09
120	29.12 ± 3.05	29.50 ± 4.89	9.75 ± 1.03

[a]The activities of different enzymes have been tested from the exiting perfusate. ALAT, alanine amino transferase; ASAT, aspartate amino transferase; CPK, creatinin phosphokinase. Normal values ALAT – ASAT were 0–46 UI/l (ref. IFFA-Credo). There was no difference in the values obtained from isolated rat liver perfused with minimal perfusion medium, TC or TC + PC vesicles. Therefore, each value represents the mean ± SEM of both livers tested (n = 12).

in situ BS synthesis. The measurement of nearly normal BS/PC molar ratios in control animals perfused for 40 min after isolation would mean that BS-dependent bile secretion is sustained at a very low level and that the liver PC pool is far from exhausted. As stated in Materials and Methods, only about 2% of the total liver PC pool was eliminated by animals in groups 2 and 3 during the 2-hr study period. Thus, within a short period, bile secretion in controls became largely BS-independent. The maintenance of this type of secretion was formerly the only bile criteria for liver viability. By simulating normal enterohepatic circulation, addition of BS to the perfusate activates secretion of PC and BS in the bile. In this respect, i.e., normalization of the lipid content of secreted bile, TC perfusion with or without PC unilamellar vesicles had the same effect in our concentration conditions. Judging from the levels of ALAT, ASAT and CPK measured in the hepatic perfusate effluent, enzyme activity remains in the normal range during the 2-hr perfusion period. Unlike Krell et al. (34) and Sugano et al. (35), who reported a 50% decrease in bile flow after 40 and 70 min of perfusion, we observed a stable flow; this observation is strong evidence for the viability of our model. Given the level of the β-glucuronidase activity, the hepatic detoxification process would appear to remain intact. This finding was further supported by the measurement of normal osmolarity in exiting perfusate, which is an indication of limited cell damage. It previously has been demonstrated that a hemoglobin-free perfusate can be used without affecting the viability of the liver; Sugano et al. (35) showed that perfusion of a saline bicarbonate mixture without macromolecules at a flow rate of 3–3.5 ml/min/g of liver was adequate to preserve the fine structure of the entire liver for 70 min. To prolong viability by maintaining normal hydrostatic pressure and preventing cellular distension, the perfusate used in this study was supplemented with dextran (mol wt 70,000), which does not interfere with TC and PC.

To avoid the depletion of the perfusate and thus better duplicate the consistent quality of the portal flow, the perfusate was not recirculated. In addition to simplifying the experimental technique and avoiding artefacts due to perfusate variations, the unrecirculated mode has two main experimental advantages. First, exiting perfusate can be collected and used to investigate any number of parameters. The second benefit of not recirculating the perfusate is a great improvement in the reproducibility of results. In our experiments, clearance of TC by the liver was highly efficient since it was 98 and 95% in animals perfused with or without PC unilamellar vesicles. Assays carried out with ^{14}C-TC showed that uptake was almost immediate, since all the label was taken up within 20 sec. At the end of the experiment, no label was even detected in the liver of experimental animals of groups 2 and 3, thus indicating that clearance of TC was practically complete. A rapid transfer of TC from perfusate to bile is shown by the fact that 90% of the label of the perfused TC is secreted in the bile within 20 min. With regard to uptake, excretion and kinetics, this model behaves identically to the in vivo model using desoxycholate (unpublished data). Uptake of BS, which is practically unsaturable under physiological conditions, seems to be independent of hydroxylation and conjugation (36). The addition of TC with or without PC unilamellar vesicles to the perfusate did not affect uptake and secretion of BS. With or without PC unilamellar vesicles in our conditions, BS reach the liver as monomers and dimers; the incorporation of TC into the lipid bilayer interfacial phase of the PC unilamellar vesicles is negligible (13,24).

The model proposed presents several advantages for use in pharmacological and physiological studies. These advantages include preservation of active bile secretion, constant bile flow, normal bile lipid secretion and excellent organ viability. To furnish a vehicle for liposoluble molecular species, PC unilamellar vesicles can be added to the perfusate without altering the quality of bile secretion.

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Activities of Liver Mitochondrial and Peroxisomal Fatty Acid Oxidation Enzymes in Rats Fed *trans* Fat

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The effect of *trans* fat on the activities of liver mitochondrial and peroxisomal fatty acid oxidation enzymes was examined in various strains of rats. When Wistar and Sprague-Dawley rats were fed for 30 days diets containing either olive oil or partially hydrogenated corn oil as a source of *cis*- or *trans*-octadecenoate, respectively, the activities of various enzymes of mitochondrial and peroxisomal β-oxidation measured with *cis*- and *trans*-9-octadecenoic acid as substrates showed little dietary fat-dependent change. In Fischer 344 rats, feeding *trans* fat for 15 mo increased only moderately various enzymes of β-oxidation except for carnitine acyltransferase. The rate of mitochondrial ketogenesis and the activity of carnitine acyltransferase measured with *trans*-9-octadecenoic acid as a substrate were about half those with the *cis*-counterpart. Peroxisomes oxidized *trans*-9-octadecenoyl-CoA at a rate comparable to the *cis*-counterpart. It was concluded from this study and previous ones that the difference in the geometry of dietary fatty acid had only a marginal effect in modulating the hepatic fatty acid oxidation system, in spite of marked differences in the metabolic behavior of *cis*- and *trans* fatty acid in cell-free preparations and perfused liver.
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Hydrogenation products of edible oil usually contain varying amounts of *trans* fatty acids, most exclusively as *trans*-octadecenoate (1–3). A number of studies have unequivocally demonstrated that liver and heart mitochondria oxidize *trans* fatty acid at a rate lower than that observed with the corresponding *cis* isomer (4–8). In addition, we have recently observed that the peroxisomal β-oxidation system is also able to discriminate the geometrical difference of fatty acids (9).

There is a possibility that dietary *trans* fat and *cis* fat exert different effects on the fatty acid oxidation pathway. In some studies, dietary *trans*-octadecenoate affected the activities of mitochondrial (5,10) and peroxisomal β-oxidation (11,12). The results obtained in these studies, however, were inconclusive, since the *trans* effect was compared inappropriately among fat sources in which the difference in the fatty acid composition was not confined to the geometrism alone. In addition, little is known about the effect of *trans* fat on the activities of respective enzymes in mitochondrial and peroxisomal β-oxidation. In this context, activities of key enzymes involved in fatty acid oxidation were compared currently in various strains of rats fed either *trans* or *cis* fat. The fatty acid composition of these dietary fats was made similar except for the difference in the geometrical configuration.

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MATERIALS AND METHODS

Animals and diets. In experiment 1, specific pathogen-free male Wistar (Kyudo Co., Kumamoto, Japan) or Sprague-Dawley (Seiwa Experimental Animals, Fukuoka, Japan) rats at 5 weeks of age weighing 120–150 g were housed individually in a temperature-controlled room (20–23 C) with illumination from 0800 to 2000 hr. The animals were divided into two groups of six rats each and for 30 days were fed purified diets containing either 15% olive oil or 15% partially hydrogenated corn oil supplemented with safflower oil as the source of essential fatty acid; they then were killed by decapitation. In experiment 2, the long-term effect of dietary *trans* fat was examined in male Fischer 344 rats (Shizuoka Experimental Farm, Shizuoka, Japan). The animals, 4 weeks old and weighing about 75 g, were fed purified diets containing either 10% olive oil or a 10% partially hydrogenated corn oil/safflower oil mixture for 15 mo. Fatty acid compositions of dietary fats are shown in Table 1. The two dietary fats had similar fatty acid compositions and contained octadecenoic acid as the major component, but differed in the geometrical configuration. The composition of the purified diet was the same as that described previously (13); it contained 20% casein, 1% vitamin mixture, 4% mineral mixture, 0.2% choline chloride and 2% cellulose and made up to 100 with sucrose.

Enzyme assays. The rate of ketogenesis in the heavy mitochondrial fraction of the liver was measured by the method of Ontko et al. (14,15) in which optimal concentration of Ca^{2+} (0.4 mM) was used. The liver particulate fraction containing both light and heavy mitochondria (16) was used for the measurement of palmitoylcarnitine-dependent ferricyanide reduction (mitochondrial β-oxidation) (17), cyanide-insensitive acyl-CoA–dependent NAD reduction (peroxisomal β-oxidation) (18), peroxisomal acyl-CoA oxidase (19), carnitine acyltransferase (20) and mitochondrial acyl-CoA dehydrogenase (21). Carnitine acyltransferase is primarily a mitochondrial enzyme but is located also in peroxisomes (20).

TABLE 1

Fatty Acid Composition of Dietary Fats

	Experiment 1		Experiment 2	
Fatty acid	*cis* Fat[a] (%)	*trans* Fat[b] (%)	*cis* Fat (%)	*trans* Fat (%)
16:0	9.4	11.3	9.7	13.1
18:0	2.0	7.2	3.4	7.9
t-18:1	—	45.1	—	42.0
c-18:1	77.0	26.0	74.1	27.2
cc-18:2	9.3	9.3	9.4	9.4

[a]Olive oil.
[b]Partially hydrogenated corn oil/safflower oil mixture.

Lipid analyses. Serum and liver lipids were analyzed for triglyceride, cholesterol and phospholipid as described previously (22). The fatty acid composition of mitochondrial phosphatidylcholine isolated by thin-layer chromatography (23) was determined by gas-liquid chromatography using a combination of OV-275 and DEGS columns as described previously (24).

Materials. The *cis*- and *trans*-9-octadecenoic acids, their chlorides and palmitoylchloride were purchased from Sigma Chemical Co. (St. Louis, Missouri). CoA esters of fatty acids were prepared by the method of Wieland and Rueff (25). Bovine serum albumin (fraction V, fatty acid-free) was purchased from Miles Laboratories Inc. (Elkhart, Indiana). Horseradish peroxidase and 3-hydroxybutyrate dehydrogenase came from Boehringer (Mannheim, Federal Republic of Germany). ATP, ADP, NAD and NADH were from Oriental Yeast Co. (Tokyo, Japan). Otsuka Pharmaceutical Co. (Tokushima, Japan) provided l-carnitine and palmitoylcarnitine. CoA was a gift from Kohjin Biochemicals (Tokyo, Japan).

Statistical analysis. Data were analyzed by two-way analysis of variance; differences of the pair of means were inspected at the level of $p < 0.05$ (26).

RESULTS

Effect of short-term feeding of trans *fat on fatty acid oxidation enzymes (experiment 1).* Table 2 shows activities of fatty acid oxidation enzymes in Wistar and Sprague-Dawley rats fed *cis* or *trans* fats for 30 days. No differences were found in food intake or growth of animals among the various groups (values not shown). Activities were measured with *cis*- and *trans*-9-octadecenoic acid or their CoA esters as substrates. The effect of *trans* fat on enzyme activities was rather marginal, and a moderate increase in cyanide-insensitive NAD reduction (peroxisomal β-oxidation) was the sole effect observed. No significant differences in the enzyme activities were noted between different strains of animals except for mitochondrial ketogenesis in one instance. The rates of ketone body formation from *trans* fatty acid were about half those from *cis* isomer in various situations and paralleled well the activities of carnitine acyltransferase. Mitochondrial acyl-CoA dehydrogenase, peroxisomal β-oxidation and acyl-CoA oxidase activities were approximately the same among different substrates.

Strain- or dietary fat-dependent changes were not confirmed in serum lipid levels, while dietary *trans* fat decreased the hepatic triglyceride level in Sprague-Dawley but not in Wistar rats (Table 3). Hepatic cholesterol contents in rats fed *trans* fat were lower than those in animals fed *cis* fat, and the difference was marked in Sprague-Dawley rats. Concentrations of liver phospholipid were approximately the same among various groups.

Table 4 shows fatty acid composition of mitochondrial phosphatidylcholine in rats fed *cis* and *trans* fat. Accumulation of *trans*-octadecenoic acid in rats fed *trans* fat was accompanied by a decrease in the content of saturated fatty acid (palmitic and stearic acids) rather than that of *cis*-octadecenoic acid. The amount accumulated was significantly higher in Wistar rats. Linoleic acid content increased and highly unsaturated fatty acids decreased in rats fed *trans* fat.

Effects of long term feeding of trans *fat on fatty acid oxidation enzymes (experiment 2).* Table 5 shows activities of fatty acid oxidation enzymes in Fischer 344 rats fed *cis* or *trans* fat for 15 mo. No differences were found in food intake or growth of animals fed different fats (27). In this experiment, activities of mitochondrial β-oxidation were measured spectrophotometrically using palmitoylcarnitine as the substrate; other enzyme activities were measured with palmitoyl-CoA as well as *cis*- and *trans*-9-octadecenoyl-CoA as substrates. As Table 5 shows, activities of peroxisomal β-oxidation and acyl-CoA oxidase were slightly but significantly increased in rats fed *trans*

TABLE 2

Activities of Fatty Acid Oxidation Enzymes in Wistar and Sprague-Dawley Rats Fed *cis* and *trans* Fat for 30 Days

	Enzyme activities (nmol/min/mg protein)			
	Substrate (Wistar rats)		Substrate (Sprague-Dawley rats)	
Dietary fats and enzymes	*c*-9-18:1	*t*-9-18:1	*c*-9-18:1	*t*-9-18:1
cis Fat				
Mitochondrial ketogenesis	7.81 ± 1.20[a,b]	3.62 ± 0.76[c]	5.05 ± 0.43	2.59 ± 0.36[c]
Acyl-CoA dehydrogenase	26.5 ± 1.5	29.3 ± 2.6	26.4 ± 3.4	28.6 ± 3.8
Carnitine acyltransferase	5.60 ± 0.32	2.58 ± 0.10[c]	5.40 ± 0.12	2.67 ± 0.24[c]
KCN-insensitive NAD reduction	5.64 ± 0.50[d]	5.66 ± 0.44	5.74 ± 0.26	5.20 ± 0.19
Acyl-CoA oxidase	4.28 ± 0.47	4.17 ± 0.50	3.84 ± 0.33	3.60 ± 0.26
trans Fat				
Mitochondrial ketogenesis	6.44 ± 0.81	3.08 ± 0.58[c]	5.62 ± 0.83	2.88 ± 0.51[c]
Acyl-CoA dehydrogenase	29.6 ± 1.2	33.2 ± 2.0	30.9 ± 1.6	34.7 ± 2.4
Carnitine acyltransferase	6.50 ± 0.44	3.01 ± 0.34[c]	6.12 ± 0.62	2.58 ± 0.42[c]
KCN-insensitive NAD reduction	7.55 ± 0.84	7.14 ± 0.78	6.45 ± 0.69	6.22 ± 0.64
Acyl-CoA oxidase	4.56 ± 0.48	4.53 ± 0.42	4.55 ± 0.41	4.20 ± 0.38

[a]Means ± SE of 6 rats.

[b]Significantly different from the corresponding value for Sprague-Dawley rats at $p < 0.05$.

[c]Significantly different from the corresponding value with c-9-18:1 at $p < 0.05$.

[d]Significantly different from the corresponding value in rats fed *trans* fat at $p < 0.05$.

TABLE 3

Serum and Hepatic Lipid in Wistar and Sprague-Dawley Rats Fed *cis* and *trans* Fat for 30 Days

Dietary fats and strains	Serum lipids (mg/dl)			Hepatic lipids (mg/g)		
	Triglyceride	Cholesterol	Phospholipid	Triglyceride	Cholesterol	Phospholipid
cis Fat						
Wistar rats	549 ± 119[a]	105 ± 11	256 ± 20	12.0 ± 1.2[b]	2.45 ± 0.15[b]	28.2 ± 1.0
Sprague-Dawley rats	492 ± 110	95.2 ± 5.6	260 ± 9	38.5 ± 3.6[c]	3.70 ± 0.50[c]	25.9 ± 0.6
trans Fat						
Wistar rats	546 ± 99	94.8 ± 2.0	256 ± 14	10.6 ± 1.6	1.99 ± 0.14	29.7 ± 1.3
Sprague-Dawley rats	431 ± 84	92.7 ± 7.8	233 ± 17	14.2 ± 1.3	2.18 ± 0.11	27.0 ± 0.7

[a]Means ± SE of 6 rats.

[b]Significantly different from the corresponding value in Sprague-Dawley rats at $p < 0.05$.

[c]Significantly different from the corresponding value in rats fed *trans* fat at $p < 0.05$.

TABLE 4

Fatty Acid Composition of Mitochondrial Phosphatidylcholine in Wistar and Sprague-Dawley Rats Fed *cis* and *trans* Fat for 30 Days

Dietary fats and strains	Fatty acid (wt %)								
	16:0	*t*-16:1	*c*-16:1	18:0	*t*-18:1	*c*-18:1	*cc*-18:2	20:4	22:6
cis Fat									
Wistar rats	19.0 ± 0.3[a,b]	—	1.4 ± 0.1	19.8 ± 0.6[b]	—	13.9 ± 0.5[b,c]	6.6 ± 0.5[b]	27.8 ± 0.9[b,c]	6.0 ± 0.2[b]
Sprague-Dawley rats	20.0 ± 0.3[b]	—	1.6 ± 0.9	17.7 ± 0.7[b]	—	14.7 ± 0.5	7.6 ± 0.5[b]	24.8 ± 0.3[b]	5.5 ± 0.1[b]
trans Fat									
Wistar rats	17.4 ± 0.5	1.4 ± 0.0[c]	1.4 ± 0.1	11.9 ± 0.4	12.0 ± 0.6[c]	11.4 ± 0.3	11.3 ± 0.5[c]	23.7 ± 0.7[c]	4.0 ± 0.1[c]
Sprague-Dawley rats	18.4 ± 0.5	0.9 ± 0.2	1.8 ± 0.2	11.8 ± 0.7	8.9 ± 0.5	14.2 ± 0.4	12.9 ± 0.4	19.9 ± 0.6	3.2 ± 0.3

[a]Means ± SE of 6 rats.

[b]Significantly different from the corresponding value in rats fed *trans* fat at $p < 0.05$.

[c]Significantly different from the corresponding value in Sprague-Dawley rats at $p < 0.05$.

TABLE 5

Activities of Fatty Acid Oxidation Enzymes in Fischer 344 Rats Fed *cis* and *trans* Fat for 15 Months

Dietary fats and enzymes	Enzyme activities for given substrates (nmol/min/mg protein)		
	16:0	*c*-9-18:1	*t*-9-18:1
cis Fat			
Mitochondrial β-oxidation	6.21 ± 0.54[a]	—	—
Acyl-CoA dehydrogenase	9.84 ± 0.42[b,c]	7.25 ± 0.44[b]	7.09 ± 0.44[b]
Carnitine acyltransferase	4.26 ± 0.32[c]	2.76 ± 0.15[c]	1.10 ± 0.09[c]
KCN-insensitive NAD reduction	8.83 ± 0.49[b,c]	6.33 ± 0.67[b]	7.69 ± 0.54[b]
Acyl-CoA oxidase	3.18 ± 0.18[b,c]	2.46 ± 0.13[b]	2.41 ± 0.28[b]
trans Fat			
Mitochondrial β-oxidation	7.77 ± 0.81	—	—
Acyl-CoA dehydrogenase	12.8 ± 1.0[c]	9.54 ± 0.73	9.60 ± 0.72
Carnitine acyltransferase	4.80 ± 0.37[c]	3.18 ± 0.22[c]	1.37 ± 0.14[c]
KCN-insensitive NAD reduction	11.0 ± 0.5[c]	8.57 ± 0.58	9.96 ± 0.78
Acyl-CoA oxidase	4.31 ± 0.29[c]	3.50 ± 0.21	3.41 ± 0.22

[a]Means ± SE of 8 rats.

[b]Significantly different from the corresponding values in rats fed *trans* fat at $p < 0.05$.

[c]Significantly different from the corresponding values measured with other fatty acyl-CoA substrates at $p < 0.05$.

fat, irrespective of the difference in the fatty acid substrate. Acyl-CoA dehydrogenase but not carnitine acyltransferase and mitochondrial β-oxidation also increased significantly in rats fed *trans* fat. The activity of the various enzymes examined was highest when saturated fatty acyl-CoA was used as a substrate and was uninfluenced by the geometrical difference of octadecenoyl-CoA substrates, except for a significantly low value for carnitine acyltransferase with the *trans* counterpart.

Consistent with the preceding experiments, concentrations of hepatic cholesterol, serum cholesterol and phospholipid were lower in rats fed *trans* fat than those fed *cis* fat (data have been reported elsewhere [27]). Although the fatty acid composition of hepatic organelles was not determined in this experiment, examination of the fatty acid compositions of serum lipids (27) revealed the same line of changes in rats fed *trans* fat as observed in experiment 1.

DISCUSSION

A wealth of experiments done with isolated heart or liver mitochondria has demonstrated a distinctly lower rate of oxidation of *trans*-octadecenoic acid compared to the *cis* counterpart. We have shown previously that *trans*-9-octadecenoic acid is oxidized to ketone bodies at a rate about one-half to one-third that found with the *cis* counterpart in liver mitochondria, and the response parallels well that of the activities of carnitine acyltransferase measured with *cis*- and *trans*-9-octadecenoyl-CoA as substrates (9). These results were confirmed in the present study (Tables 2 and 5). It is thus plausible that the substrate specificity of carnitine acyltransferase is responsible for the impaired rate of oxidation of the *trans* fatty acid in isolated mitochondria.

Although the rates of peroxisomal β-oxidation were approximately the same for *cis*- and *trans*-9-octadecenoic acids (Tables 2 and 5) (9,28), we recently demonstrated that the affinity of the *trans*-acyl-CoA toward acyl-CoA oxidase was considerably lower than that of the *cis* counterpart (9). Thus, the fatty acid oxidation systems not only in mitochondria but also in peroxisomes seem to oxidize the *trans* acid less efficiently.

The mitochondria have very limited ability to oxidize monosaturated fatty acids with 22-carbons, apparently due to the substrate specificity of acyl-CoA dehydrogenase. When rats were fed a diet rich in docosadienoic acid (22:1) such as in rapeseed oil and hydrogenated fish oil, however, a striking increase in the activity of peroxisomal β-oxidation occurred (11,12,28,29). This type of compensatory mechanism may not operate in the hepatic fatty acid oxidation system of rats fed *trans*-octadecenoate fat, since activities of enzymes involved in the systems were virtually uninfluenced by the geometry of dietary fats.

Several studies have examined the effect of *trans*-octadecenoic acid on the activities of the fatty acid oxidation pathway (6,10–12). However, these results are hard to evaluate as the *trans* effect was compared inappropriately, i.e., between hydrogenated fat and unhydrogenated fat from which hydrogenated products were prepared; thus the difference in the fatty acid composition was not confined to geometrism. If the effect of *trans* fat on the fatty acid oxidation enzyme is compared with the fat source with the difference of geometrical configuration as a sole variable, as in the present experiment, the results can be regarded as a reflection of the geometrical effect of dietary fat. It is possible that olive oil and corn oil contain different minor components that might cause differences in enzyme activities. In this context, the *trans* effect with regard to cholesterol metabolism was reproduced even when camellia oil, with a fatty acid composition similar to olive oil, was used as the control *cis* fat (30).

In experiments in which Wistar and Sprague-Dawley rats were fed dietary fats with different configurations, the *trans* effect, although moderate, was observed only in the peroxisomal β-oxidation on one occasion, and no other changes were confirmed in various fatty acid oxidation enzymes. In a long-term feeding study with Fischer 344 rats, *trans* fat increased the activities of various fatty acid oxidation enzymes except for carnitine acyltransferase; however, the increases were rather moderate. Thus, it could be concluded that the geometry of dietary fat has only a marginal effect in modulating hepatic fatty acid oxidation enzymes, in spite of a marked difference in the metabolic behaviors of *cis* and *trans* fatty acids in the cell-free preparations (Tables 2 and 5) (4–9) and the isolated perfused liver (9,23).

We have observed previously an elevation of triglyceride content in Sprague-Dawley rats fed high (20%) but not low (5%) dietary levels of *cis* fat (31) in relation to the corresponding levels of *trans* fat. In the present study, hepatic triglyceride accumulation was observed in Sprague-Dawley but not Wistar rats fed 15% *cis* fat (Table 3). Although the causes for the observed strain-dependent responses are not clear at present, the experiment with isolated perfused liver indicated a slight difference in the metabolic fate of *cis*-octadecenoic acid between Sprague-Dawley and Wistar rats (9). The effect of the difference in the absorbability of *cis* and *trans* fats (30) may not be disregarded.

The changes observed in the concentration of hepatic cholesterol and the fatty acid composition of mitochondrial phospholipids after feeding *trans* fat were consistent with reported data (24,30–32). Dietary *trans* fat apparently exerted its effect on the metabolism of cholesterol and polyunsaturated fatty acids without seriously influencing the activities of fatty acid oxidation enzymes.

We have compared previously the metabolic fate of *cis* and *trans* fatty acids in isolated perfused livers from Wistar and Sprague-Dawley rats, and found a marked strain-dependent difference in the fate of *trans* fatty acid (9). In addition, the fatty acid oxidation pathway had only a minor role in determining the metabolic rate of *trans* fatty acid in perfused rat liver; rather, the esterification pathway was primarily responsible in this context (9). In cases where the fatty acid oxidation pathway is not an important determinant for the metabolic fate of *trans* fatty acid, the activities of fatty acid oxidation enzymes may not necessarily respond to dietary *trans* fat, as observed in the present study.

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Epicuticular Waxes of Maize as Affected by the Interaction of Mutant *gl8* with *gl3*, *gl4* and *gl15*

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Chemical composition of epicuticular waxes from double mutants of maize *gl2gl8*, *gl3gl8*, *gl4gl8* and *gl15gl8* is compared to that of the wild type and of the single mutants. The wax composition was moderately affected in the double mutants studied. Meanwhile, free fatty acids became a normal class constituent of the waxes. The sites of action of the single mutants as deduced from previous studies are confirmed. The influence of *gl2*, *gl3* and *gl4* on the terminal steps of the chain elongation process in *gl2gl8*, *gl3gl8* and *gl4gl8* genotypes is presented and discussed. The study of *gl3gl8* waxes also confirms that the mutant *gl3* induces a metabolic defect definitely different from those of *gl2* and *gl4*. The pattern of alkanes from *gl15gl8* is unusual, supporting the thesis that *gl15* controls mainly alkane synthesis. However, based on variations induced on wax composition, its manner of action is difficult to account for. Taken together, the available data on single and double mutants affecting wax synthesis in maize suggest that elongases might be heteromeric enzymes.
Lipids 22, 11-16 (1987).

In *Zea mays* plants, the leaf cuticle is typically endowed with wax deposits that decrease in amount with plant aging (1,2). These waxes are synthesized by more than one elongation-decarboxylation (ED) system (reviewed in ref. 3): ED-I, active only at the seedling stage, yields long chain compounds accumulated as alcohols, aldehydes and alkanes and may be split into an early (ED-Ia) and a late (ED-Ib) group of reactions; ED-II, effective throughout the plant's life, is mainly involved in the synthesis of esters.

The biosynthesis and deposition of the epicuticular wax layer in maize is controlled by at least 13 independent genes (4). Several mutations, including *gl8*, influence the metabolic steps leading to the synthesis of or controlling the precursors entering the ED pathways (5-9). Other mutations, such as *gl2*, *gl3* and *gl4*, control the elongation steps of ED-I. In particular, *gl3* affects the step from C_{28} to C_{30}, while *gl2*, *gl4* and *gl16* affect C_{30} to C_{32} (5,6,8). *Glossy-15* has been found to be the only mutation interfering with the ED-II system, showing a metabolic block effective between C_{18} and C_{22} (8).

This paper considers the epicuticular waxes from double mutants of *gl8*, namely *gl3gl8*, *gl2gl8*, *gl4gl8* and *gl15gl8*. A major aim of our research was to evaluate the modifications induced by an abnormal supply of wax precursors on the functioning of the ED pathway in the presence of step specific elongation mutations. The data obtained have permitted us to deduce the sites of action of single *gl* mutants in more detail.

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MATERIALS AND METHODS

Double mutants *gl2gl8*, *gl3gl8*, *gl4gl8* and *gl15gl8* were obtained in the background of the inbred line WF9 by crossing single mutants and reselecting by test-crossing plant homozygous for both mutations. Maize seedlings were grown in the greenhouse and collected at the stage of fifth leaf.

The epicuticular waxes were extracted by immersion for 45-60 sec in $CHCl_3$, and their composition was analyzed by thin layer chromatography (TLC) as previously described (10). Individual wax components were fractionated by column chromatography and identified by spectroscopic techniques as already reported (5,10-12). Gas chromatography (GC) analyses of the purified fractions were performed with a 15 m OV1 capillary column (0.3-0.32 mm id; 0.1:0.15 μm film thickness) on a HRGC Carlo Erba gas chromatograph with flame ionization detector, connected to a Spectra Physics SP4100 computing integrator for automatic data processing. Cold on-column injection mode was adopted. Hydrogen was used as the carrier gas; air and hydrogen streams were adjusted to yield optimum separations. Temperature was programmed from 60 to 170 C at 40 C/min, then to 280 C at 5 C/min, with 7 min of final isotherm. The detector port was maintained at 300 C. Alcohols were converted into their trimethylsilyl (TMS) ether derivatives using N,O-bis (trimethylsilyl) acetamide, while acids were analyzed as their methyl (Me) esters as described previously (10). A modified GC program was used to analyze intact esters: starting temperature was set at 90 C, increased up to 280 C at 40 C/min, then to 350 C at 5 C/min and held for 15 min. The detector temperature was 390 C.

Transmethylation of the esters was also carried out to yield their alcohol moieties and the Me esters of their fatty acid moieties (10), which were separated by preparative TLC in $CHCl_3$. Alcohols from ester-transesterification were then transformed into the corresponding TMS ether derivatives before GC analysis. Alkanes and aldehydes were analyzed without any derivatization.

RESULTS

Overall wax composition. Table 1 shows the percentages of the components and the relative yields of the waxes found on *gl2gl8*, *gl4gl8*, *gl3gl8* and *gl15gl8* seedlings compared to their parent single mutants and to the wild type. Worthy of note is the high total yield of waxes of *gl2gl8*, *gl4gl8* and *gl3gl8* (75, 83 and 57% of *Gl*, respectively) compared to their parent mutants, which contrasts with the results of a previous study on other double mutants (13). A similar overproduction of wax was found for an albino strain of maize (10). As in that case, this finding is probably due to the smaller size of the double mutant seedlings, which appeared covered by a more concentrated and thicker layer of wax. In association with *gl15*, *gl8* induced instead a decrease of the wax yield to 36% of the wild type.

TABLE 1

Composition (%) and Relative Yields of Epicuticular Waxes from Wild Type *(Gl)* and Single and Double Glossy Mutants *(gl)* of Maize

Components	*Gl*	*gl8*	*gl2gl8*	*gl2*	*gl4gl8*	*gl4*	*gl3gl8*	*gl3*	*gl15gl8*	*gl15*
Alkanes	1	1	8	12	4	t	8	3	3	5
Esters	16	38	36	54	36	42	26	41	57	20
Aldehydes	20	12	14	10	7	16	11	9	8	20
Alcohols	63	49	30	24	42	42	42	47	30	55
Acids	t	—	12	—	11	—	13	—	2	—
Relative yield of wax (% of Gl)[a]	100	30	75	20	83	44	57	27	36	76

—, Not detected; t, traces (≤0.5%).

[a]The data were based on wax yield per 1000 g of fresh weight of seedlings (3).

TABLE 2

Composition (%) of Alkanes of Epicuticular Waxes from Wild Type *(Gl)* and Single and Double Glossy Mutants *(gl)* of Maize

Number or carbon atoms	*Gl*	*gl8*	*gl2gl8*[a]	*gl2*	*gl4gl8*	*gl4*	*gl3gl8*	*gl3*	*gl15gl8*	*gl15*
19–25	7	17	18	1	12	1	22	3	t	18
26	t	2	1	t	1	t	1	1	5	7
27	13	10	27	23	13	15	16	15	7	11
28	t	2	3	4	2	2	2	1	13	5
29	29	43	39	64	53	70	41	42	14	25
30	t	t	1	t	1	3	1	2	14	3
31	49	26	4	4	14	8	15	32	16	26
32	t	—	1	2	1	1	1	1	13	t
33	2	—	t	t	3		1	3	10	5
34				2				t	8	

—, Not detected; t, traces (≤0.5%).

[a]Two peaks, amounting to 6% of the total are present in the chromatogram with rt smaller than those of C_{29} and C_{31}.

Double mutant *gl2gl8* appeared similar to *gl8* when percentages of esters and aldehydes are considered. On the other hand, percentages of alkanes and alcohols were more consistently similar to *gl2*. The *gl4gl8* did not show remarkable differences from either single parent mutant, apart from a clear decrease of aldehydes (from 12.16% to 7%). Esters were the most abundant compounds in *gl15gl8* wax. All double mutants waxes contained certain amounts of free fatty acids (2 to 13%), which were not present in their parent mutants.

Alkanes. The composition of the alkanes is presented in Table 2. In all cases but *gl15gl8*, the homologue C_{29} predominated, varying from 39 to 53%. The C_{31} alkane, which was the major homologue (49%) in *Gl* wax and accounted for 26% in *gl8*, represented only 4% of *gl2gl8* alkanes, a value identical to that of *gl2*. Discrete amounts of the same chain length were found in *gl4gl8* (14%) and *gl3gl8* (15%). The distribution of chain lengths among *gl15gl8* alkanes was very peculiar in that odd and even homologues were present in the range from C_{26} to C_{34}, with no significant difference in their relative amounts.

Aldehydes and alcohols. Aldehydes and alcohols from *Gl*, *gl8* and *gl15* genotypes were typically characterized by the C_{32} as dominant chain length. A reduction of the C_{32} homologue compensated for by an increase in the relative importance of the C_{30} chain length, which becomes the most abundant component, was observed in *gl2* and *gl4* (Table 3; see also 5,8). No relevant difference in the chain length distributions was evident for aldehydes and alcohols from *gl15gl8* when compared to their parent mutants and the wild type. In contrast, in *gl2gl8* and *gl4gl8*, the influence of *gl2* and *gl4* predominated over that of *gl8*, as proved by the aldehyde and alcohol compositional percentages (Table 3). *Glossy-4* mutation was less effective than *gl2* in reducing C_{32} accumulation, which accounts for 30% in *gl4gl8* aldehydes and 25% in *gl4gl8* alcohols. Almost equivalent amounts of C_{30} and C_{32} homologues were found among aldehydes and alcohols from *gl3* wax. In *gl3gl8*, the C_{32} aldehyde contributed for 75% of this fraction, whereas the C_{32} alcohol represented 34%. As for the single mutant *gl3*, the alcohols from *gl3gl8* were spread over the C_{24} to C_{32} chain lengths.

Esters. Table 4 presents the composition of the alcohol and acid moieties of the esters. In *gl2gl8*, shorter esterified alcohols having 16 to 24 carbon atoms accounted for almost the same relative amounts as in *gl8*. The long chain C_{32} alcohol, which represents 100% in *Gl*, 9% in *gl8* and 1% in *gl2*, was not present in the double mutant, whereas the C_{30} homologue, dominant in *gl2*, only amounted to 4%. A different spectrum of esterified alcohols was found in *gl4gl8*: C_{30} was 32% and C_{32} 10% of the total. In this double mutant, moreover, the distribution

TABLE 3

Composition (%) of Aldehydes and Alcohols of Epicuticular Waxes from Wild Type *(Gl)* and Single and Double Glossy Mutants *(gl)* of Maize

Number of carbon atoms	Aldehydes										Alcohols									
	Gl	*gl8*	*gl2gl8*	*gl2*	*gl4gl8*	*gl4*	*gl3gl8*	*gl3*	*gl15gl8*	*gl15*	*Gl*	*gl8*	*gl2gl8*	*gl2*	*gl4gl8*	*gl4*	*gl3gl8*	*gl3*	*gl15gl8*	*gl15*
16–22															6					
24													2	1	2		11	7		
26				1				1				2	7	4	4		19	29	1	
28	1	12	14	38	6	6	t	13	2			t	34	21	8	6	12	16	2	
29					1	—														
30	3	8	75	57	62	79	25	44	10		1	5	53	72	55	79	24	27	8	
31					1	—														
32	96	80	11	4	30	15	75	42	88	100	99	93	4	2	25	15	34	21	89	100

—, Not detected; t, traces (≤0.5%).

TABLE 4

Composition (%) of Esterified Alcohols and Acids of Epicuticular Waxes from Wild Type *(Gl)* and Single and Double Glossy Mutants *(gl)* of Maize

Number of carbon atoms	Esterified alcohols										Esterified acids									
	Gl	*gl8*	*gl2gl8*	*gl2*	*gl4gl8*	*gl4*	*gl3gl8*	*gl3*	*gl15gl8*	*gl15*	*Gl*	*gl8*	*gl2gl8*	*gl2*	*gl4gl8*	*gl4*	*gl3gl8*	*gl3*	*gl15gl8*	*gl15*
16		2	4			t				14		13	15	3	18	8	13	15	5	5
18		4	3	t		2				38		11	19	4	13	8	12	7	10	4
20		16	22	10	8	11	3	9	5	t	1	35	33	11	25	25	38	24	56	18
22		19	16	5	9	14	6	5	18	2	15	28	27	12	30	24	29	16	13	32
24		36	31	17	22	28	38	52	55	5	49	10	3	10	10	15	8	24	16	30
26		9	10	7	12	10	22	23	15	2	27	2	1	13	4	11	t	13	t	11
28		3	10	12	7	11	8	5	—	t	6	1	2	43		9	t	1	t	t
30	t	2	4	48	32	20	12	4	—	—	2			4		t		t		
32	100	9		1	10	4	11	2	7	39	t									

—, Not detected; t, traces (≤0.5%).

of C_{20}–C_{28} chains was more similar to that of *gl4*. Higher amounts of C_{30} and C_{32} esterified alcohols than in the parent mutants were found in *gl3gl8*. Moreover, both in *gl3gl8* and *gl4gl8* the distribution of ester alcohol moieties was characterized by C_{24} and C_{26} as most prominent chains. The pattern of esterified alcohols from *gl15gl8* differs from those of the parent mutants: C_{22} (18%), C_{24} (55%) and C_{26} (15%) were present in the largest amount, while the C_{20} homologue only accounted for 5%. Unexpectedly, C_{16} and C_{18} chain lengths have not been found. However, similarly to *gl15*, the C_{28} and C_{30} esterified alcohols were not components of the esters from *gl15gl8*, while C_{32} contributed 7% of the total.

The esterified acids of *gl2gl8* closely resemble those of *gl8*, with C_{16}–C_{22} accounting in total for 94% (vs 87 and 31% in *gl8* and *gl2*, respectively). A similar spectrum was found also in *gl4gl8* and *gl3gl8*. The distribution of the ester acid moieties of *gl15gl8* was peculiar in having 56% of the C_{20} homologue. As a consequence, compared to *gl8* and *gl15*, the total amount of C_{22}–C_{28} chain lengths appeared greatly reduced.

Composition of the intact esters is shown in Table 5. Slight discrepancies are observed between their chain distribution and the expected composition from a random esterification of their alcohol and acid moieties shown in Table 4.

Acids. Acids of *Gl* consisted of all the chain lengths from C_{16} to C_{32}, with high amounts of the shorter C_{16} (25%) and C_{18} (13%), followed by C_{24} (14%), C_{26} (22%) and C_{28} (12%) (Table 6). The *gl2gl8* was characterized by the predominance of the C_{16} and C_{28} chains. Acids C_{26} (12%) and C_{28} (27%) were instead the most abundant in *gl4gl8*, whereas C_{22} (38%) was the largest homologue in *gl3gl8*. Compared to the other double mutants, *gl15gl8* was characterized by large amounts of shorter homologues in the range from C_{16} to C_{24}.

DISCUSSION

The enzyme systems carrying out elongation of palmitic acid to the long fatty acyl chain (C_{20}–C_{34}) components of the epicuticular lipids are known as elongases. The elongated acyl chains can then enter different reaction pathways: (i) decarboxylation to alkanes; (ii) reduction to aldehydes and primary alcohols; (iii) release as free acids; and (iv) esterification to yield esters.

TABLE 5

Composition (%) of Esters of Epicuticular Waxes from Wild Type *(Gl)* and Single and Double Glossy Mutants *(gl)* of Maize

Number of carbon atoms	*Gl*	*gl8*	*gl2gl8*	*gl2*	*gl4gl8*	*gl4*	*gl3gl8*	*gl3*	*gl15gl8*	*gl15*
38		1	t	2				1		
40		6	2	7	3	1	6	9	1	
41		—	t	t	t	t	t	t	t	
42	t	12	7	9	6	4	16	15	8	1
43	t	t	t	t	t	t	t	1	1	t
44	t	19	13	13	11	8	29	22	21	10
45	t	t	t	t	t	t	t	1	1	t
46	1	15	20	11	15	9	26	21	18	16
47	t	t	t	t	t	t	t	t	1	t
48	3	12	9	7	8	6	15	13	10	13
49	—	t	t	t	t	t	t	t	t	t
50	2	6	6	5	6	7	6	8	7	5
51	—	t	t	t	t	t	t	t	t	—
52	6	8	14	5	10	12	2	6	7	6
53	—	t	t	t	—	t	—	—	t	—
54	32	11	7	6	11	17	t	2	9	16
55	—	t	t	t	—	—		—	t	—
56	39	8	12	12	13	19		1	10	22
57	—	t	t	1	—	t		—	1	—
58	12	2	10	19	14	13		t	5	11
59	—			t	—	t			t	
60	5			3	3	4				

—, Not detected; t, traces (≤0.5%).

TABLE 6

Composition (%) of Acids of Epicuticular Waxes from Wild Type *(Gl)* and Single and Double Glossy Mutants *(gl)* of Maize

Number of carbon atoms	*Gl*	*gl2gl8*	*gl4gl8*[a]	*gl3gl8*	*gl15gl8*
16	25	21			22
18	13	8			10
20	4	1	6		11
21			6		
22	6	3	t	38	26
23			6		
24	14	3	7	26	10
25			5		
26	22	8	12	24	6
27			—		
28	12	51	27	12	8
29					
30	t	5			4
31					
32	4				3

—, Not detected; t, traces (≤0.5%).

[a]Two peaks amounting to 31% of the total are present in the chromatogram with rt about equal to those of C_{27} and C_{29}.

In several plant species different elongation systems exist (14–18). For instance, two acyl-CoA elongases, of C_{18}-CoA and C_{20}-CoA, recently have been obtained from epidermal cell microsomes of *Allium porrum* L. (17,18).

In maize, the exploitation of the effects of several *glossy* mutations, inhibitors, light and age of the plant in the study of the epicuticular wax formation and deposition made clear the existence of at least two elongating complexes, called ED-I and ED-II (Figure 1; 3,5–11,19–21).

Level of substrate availability for ED-I and ED-II has been shown to be under genetic control, as evidenced by the effect of *gl8*, *gl1*, *gl7* and *gl18* mutations (6,8), which

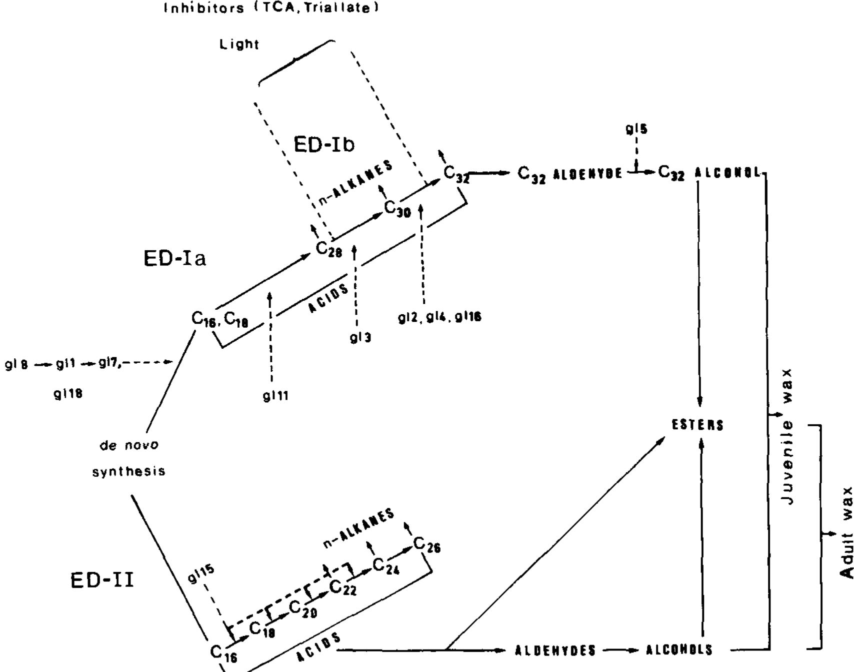

FIG. 1. Biosynthetic scheme of maize surface wax as assessed by studies on the effect of mutations, light, plant age and inhibitors on its composition (3).

resemble the *cer-p*[37] mutant of barley (22), in showing lower accumulation of wax components but unchanged chain length distributions. Results from the study of maize mutant *gl11* sustain moreover the existence of two major groups of reactions in ED-I: ED-Ia, for C_{24}-C_{28} fatty acyl chains, and ED-Ib, which further elongates these chains up to 32 carbon atoms (9).

Mutants *gl2* and *gl4* disclose defective elongation steps in ED-Ib, with a specific block at the C_{30}-C_{32} elongation step (5,8). Combination of each of them with *gl8* into double mutants is expected to produce similar modifications. Genotypes *gl2gl8* and *gl4gl8* show in fact comparable patterns in the distribution of wax classes. As a direct consequence of the metabolic blocks, an accumulation of free fatty acids is evident in the double mutants. This result discloses that in maize the release of acids from the elongating complex is possible as found in other plants (23). Evidently in the wild type waxes it is the high efficiency of maize elongating, decarboxylating, reductive and esterifying systems that prevents the accumulation of free fatty acids.

As in the single mutants, the combined synthesis of alkanes, aldehydes and alcohols deriving from the ED-I complex is much lower in the double mutants *gl2gl8* and *gl4gl8* than in the wild type. Moreover, the influence of *gl2* and *gl4* on the terminal steps of the chain elongation process is confirmed. Although mutation *gl8* differs only quantitatively from the wild type showing a good synthetic activity for the C_{32} chain length (C_{31} for the alkanes), in the two double mutants there is no evidence for any restoration of the activity for the synthesis of those chain lengths, prevailing the biochemical defect brought about by *gl2* and *gl4*. Other mutations are known in plants that affect the intermediate or final steps of the chain lengthening process (15). In maize, besides *gl2* and *gl4*, a third mutation, *gl16*, was found to control the C_{30}-C_{32} elongation step (3). The finding of those three mutants, mapping at different genetic *loci* (24), reveals a very complex biochemical regulation of wax biosynthesis and suggests that elongases might be heteromeric enzymes.

In *gl2gl8* and *gl4gl8* the ED-II system appears unaffected: esters, in fact, account for almost the same amount as in *gl8* with a very similar composition. This was expected, considering that single mutants *gl2* and *gl4* affect ED-Ib and *gl8* influences only substrate availability of wax precursors.

Mutation *gl3* controls the ED-Ib pathway at the C_{28}-C_{30} elongation step, leading to the accumulation in comparable amounts of C_{29}, C_{31} alkanes and C_{30}, C_{32} aldehydes and alcohols (6). In the double mutant *gl3gl8*, the precursor acyl chains are abnormally supplied (*gl8* effect); they, moreover, are utilized by ED-Ib at a low rate (*gl3* effect) and probably are forced to enter associate pathways or to be released from the elongase(s). In fact, as in *gl2gl8* and *gl4gl8*, combination into a double mutant of *gl8* and *gl3* results in an accumulation of free fatty acids. The homologue distribution is, however, peculiar. In *gl3gl8* free fatty acids of the shorter C_{22}, C_{24} and C_{26} chains totally account for 88%, whereas C_{28} represents 12%. The situation found in the esters further confirms the effect of *gl3* on the earliest reactions of ED-Ib. Resembling *gl3*, in fact, the esterified alcohol C_{26} accumulates in *gl3gl8* (22% vs 9% in *gl8*). No significant differences are observed in the esterified acid distribution of *gl8* and *gl3gl8*, revealing an ED-II complex normally functioning.

The biochemical regulation of the acyl chain lengthening due to *gl3 locus* must be, however, different from that of *gl2* and *gl4 loci*. Neither the single mutant *gl3* nor the double mutant *gl3gl8* evidences a very drastic block of the synthesis of the longest C_{30}, C_{32} chains associated with an accumulation of the preceding homologue, C_{28}. For instance, the amounts of the C_{30} and C_{32} esterified alcohols

in *gl3gl8* are 12% and 11%, respectively, which is much more than in the single mutants. Both of these chains are also present in discrete amounts in the free alcohol fractions, a situation resembling more closely that of *gl3*. In the aldehydes, the C_{32} homologue even accounts for 75%, whereas in *gl3* it represents 42%, suggesting that the double mutant partially restores the synthetic activity for this chain length. This observation indicates that the *gl3* metabolic defect is definitely different than those brought about by *gl2* and *gl4*.

Mutation *gl15* compared to *Gl* shows only minor modifications in wax composition. An unusual accumulation of alcohols of 16 and 18 carbon atoms incorporated into the esters is peculiar to this mutant. This was considered evidence that *gl15* affects ED-II between C_{18}–C_{22} elongation steps. Data from wax analysis of *gl15gl8* clearly indicate that neither *gl8* nor *gl15* affects the elongation processes controlled by ED-I. In the double mutant, in fact, the prevailing chains of those wax fractions have 32 carbon atoms, like the wild type and the single mutants. On the other hand, the reduced synthesis of alcohols and aldehydes induced by *gl8* in the double mutant *gl15gl8* confirms that the *gl8* mutation affects the supply of precursors entering the ED-I pathway. Moreover, in *gl15gl8*, the reduced availability of the 32 chain length for the ester synthesis, because of the low efficiency of ED-I due to *gl8*, is remarkable.

The spectrum of alkanes from *gl15gl8* is very unusual and to our knowledge unprecedented in plant waxes. Compared to the parent mutants, higher amounts of even chain alkanes are present, making up the 53% of this fraction. As a consequence, the synthesis of C_{27}, C_{29} and C_{31} alkanes, major homologues in *gl8* and *gl15*, appears reduced in the double mutant. It may be of interest to recall the data obtained from a study on the interaction between trichloroacetic acid and *gl2* that indicated the possible existence of an ED complex specific only for alkanes (19). It is evident from the data of the double mutant that *gl15* affects alkane synthesis, even if there is no obvious explanation of how the interaction of *gl8* and *gl15* should lead to the formation of even chain alkanes.

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The Major Gangliosides of the Bovine Pineal Body

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Acetone powders of fresh-frozen pineals were extracted with chloroform/methanol mixtures. By column chromatography on silicic acid, mild alkaline methanolysis, ion-exchange high performance liquid chromatography and a final thin layer chromatography on silicic acid, the major glycosphingolipids were purified from the extracts of a total of 300 bovine pineal bodies. Chromatographically purified fractions were characterized by gas chromatographic analysis. The most prominent glycosphingolipid appeared to be cerebroside. In addition, five different gangliosides were found in detectable levels. The two major gangliosides have the chromatographic and component characteristics of GD_3 and GM_3, with disialoganglioside predominating. Gangliosides indistinguishable from purchased standards of GM_1 and GD_{1a} were third and fourth, respectively, in amount. The fatty acid profiles of the two lactosyl gangliosides are similar and significantly different from those of the two gangliotetraose gangliosides. The fifth most prominent ganglioside, present at a level of 1.09% of total recovered ganglioside sialic acid, appears to be a novel trisialoganglioside, called GT_x. This new molecule has a component ratio of gal:glc:sialic acid:amino sugar of approximately 1:2:3:1. Similarities between bovine pineal and rod outer segments are discussed.
Lipids 22, 17–21 (1987).

The pineal body is unique in the mammalian central nervous system in that its innervation may be synaptically homogeneous (2). These synaptic endings appear noradrenergic, but they also contain large amounts of serotonin (3). Excised pineals have a relatively large fraction of total volume as synaptic endings (3), and the bulk of the remainder is composed of the pinealocytes themselves. Pinealocytes contain a high concentration of serotonin as the precursor for melatonin, which is the major hormonal product of the pineal body (4). The presence of serotonin in the synaptic endings may be due to "leakage" from pinealocytes, with subsequent uptake into nerve terminals (3). Therefore, excised pineals are enriched in membranes that interact with two major neurotransmitters, noradrenaline (norepinephrine) and serotonin.

Glycoconjugates are localized in membranes, and there is evidence that some of them, such as gangliosides, are concentrated in synaptic membranes (5,6). There is additional evidence that some gangliosides may be evenly distributed on neuronal membranes, and that some predominate in cells other than neurons (7,8). Recently, however, a particular ganglioside has been indicated as a marker for mouse brain synapses (9). Knowledge of the gangliosides of pineal might provide important evidence for hypotheses about the many membrane-associated activities of noradrenaline and serotonin. Finding a membrane molecule in an unusually large concentration or finding unusual membrane molecules in pineals would be sufficient reason to study these molecules further for a possible functional role. With this approach in mind, we have examined the glycosphingolipids of fresh-frozen bovine pineals.

MATERIALS AND METHODS

Glycolipid extraction. Fresh-frozen bovine pineals (Pel-Freeze, Rogers, Arkansas) were treated with a total of 10 vol (vol/wt) of acetone after an initial hand grinding in a mortar and pestle in a small volume of acetone (A). The total mixture was stirred overnight at 4 C and filtered to yield pineal acetone powder. Glycolipids are extracted from the acetone powder by a modification of the method of Wolfe (10). The acetone powder was first stirred with 19 vol of chloroform/methanol (C/M; 2:1, v/v) at room temperature for 2 hr, followed by a 4-min, high-speed mechanical homogenation. The residue was rextracted with C/M (1:1, v/v) to which 5% water had been added, without the room-temperature stirring. Finally, the residue was extracted with C/M (1:2) with 5% water (v/v/v). The extracts were combined and evaporated. All solvents were analytical grade or better.

Silicic acid column chromatography. A modification of the procedure of Ishizuka et al. (11) was used. Pure silicic acid powder (Mallinckrodt SilicAR CC-7, 100–200 mesh) was heated at least 24 hr at 100–110 C, suspended in chloroform and poured into a glass column, using 1 g silicic acid per 25 mg of sample. Sample was applied as a suspension in chloroform, and the column was eluted batchwise. Each fraction consisted of seven column volumes of solvent mixtures with increasing polarity. The solvents are the following (all ratios by volume): 1, C; 2, A/C (1:3); 3, A/C (1:1); 4, A/C (3:1); 5, A; 6, M/C (1:3); 7, M/C (1:1); 8, M/C (3:1); 9, M.

Alkaline methanolysis. This treatment is used to break ester linkages (12), with only minor cleavage of amide and glycosidic bonds (13). The dried sample is solubilized by agitation (sonication or vigorous stirring) in a small volume (usually 15 ml) of 0.1 M methanolic NaOH and warmed at 37 C for 2 hr. The solution is taken nearly to dryness by rotary evaporation (to remove most of the methanol and to concentrate glycolipids into nondialyzable micelles) and dialyzed against two changes of distilled water at 4 C for 24 hr. The dialyzed sample is then dried by rotary evaporation. This procedure, used on an eluent fraction from the silica column, resulted in essentially complete removal of phospholipids, with nearly complete retention of glycolipids. The resulting mixtures were then further purified by HPLC.

High performance liquid chromatography (HPLC). HPLC was performed in an ion-exchange mode, using Waters Radial-Pak microBondapak NH_2 columns, 10 μm column packing, in the procedure of Whalen et al. (14).

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The ganglioside nomenclature is according to the system of Svennerholm (1): GM_3, NeuAcα2 → 3Galβ1 → 4Glc-Cer; GM_1, Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc)β1 → 4Glc-Cer; GD_{1a}, NeuAcα2 → 3Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc)β1 → 4Glc-Cer; GD_{1b}, Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc8 ← 2αNeuAc)β1 → 4Glc-Cer; GT_{1b} NeuAcα2 → 3 Galβ1 → 3 GalNacβ1 → 4 Gal(3 ← 2αNeuAc8 ← 2αNeuAc)β1 → 4 Glc-Cer.

Gangliosides are eluted from the column by a gradient of 1 M aqueous NaCl into methanol. Different individual columns result in slightly different elution times for the same ganglioside, but the variation within a single column, from run to run, is less than one minute, in a 60-min procedure. Solvents were HPLC grade, purchased from MCB (Los Angeles, California).

Thin layer chromatography (TLC). The solvent most commonly used, both for qualitative and preparative TLC, was C/M/2.5 M aqueous NH_3 (60:35:8, v/v/v) (solvent A). Glycolipids were determined on qualitative plates by spraying with the phenolsulfuric reagent, positive for carbohydrate (15). Preparative plates were exposed to iodine vapor (<3 min), followed by evaporation overnight before elution. Glycolipids were eluted from scraped-off silica by C/M/water (5:5:1, v/v/v). Thin layer plates were Brinkmann aluminum-backed plates, precoated with silica gel 60, F-254. Preparative plates were the same, except 2-mm thick, on glass. Standard glycolipids were purchased from Supelco (Bellefonte, Pennsylvania).

Gas liquid chromatographic analysis (GLC). Methanolyses were performed by the procedure of Yoshida and Mega (16). The trimethylsilyl ethers of saccharides and the methyl esters of fatty acids were initially determined by gas chromatography on a 6′ packed column, containing 2.5% SE30 on 100–200 Gas Chrom Q with nitrogen carrier gas. All quantification was done on a 30-m fused quartz capillary column, coated with SE-30 (J&E, Los Angeles, California), using a flame ionization detector. A program of 110–250 C at 4 C per minute was used. Helium at a flow rate of 2 ml/min was the carrier gas. Each methanolysis of purified, extracted lipids was quantified by comparison to samples of purchased glycolipid standards, in approximately equal amounts. Each individual saccharide and the total fatty acid components were quantified by determination of a correction factor, which was calculated to take account of the entire methanolysis procedure, as well as the GLC detector response. Purchased standards of purified gangliosides, either GM_1 or GD_{1a}, were taken through the methanolysis and GLC procedures with each group of pineal preparations, in approximately the same molar amount. Although a large series of comparisons was done, for routine purposes 50 nmol of standard was used for comparison to samples of GD_3 and GM_3. Twenty-five nmol was used for comparison to samples of isolated GD_{1a} and GM_1, and 10 nmol of standard was used for the GT_x samples. Successive determinations of a correction factor with any one amount of standard typically varied less than 5% from run to run, except for the 10 nmol standards. The 10-nmol level gave variations as high as 15%. The overall correction factor for each component was determined from an average of at least three such determinations. Standards were gas chromatographed at the beginning and end of each series of experimental samples. Glucose was used as the normalizing component in the purchased gangliosides because, in our work, it gave the most complete yield. For example, sialic acid gave, from a 50 nmol sample of purchased GM_1 standard, 68% as much peak area on the gas chromatogram as did glucose. In the pure molecule, they should be present in equimolar amounts, so a correction factor of 1.46 was used for sialic acid in the samples of GD_3 and GM_3. Because the correction factors were found to vary significantly with the amount of standard used, different correction factors were determined for each significantly different amount of sample.

Carbohydrate standards were purchased from ICN (Irvine, California). Peak identifications were made by comparison to individual carbohydrate standards, carried through the same procedure. Peak quantification was by both electronic integrator and triangulation.

Colorimetric tests. Crude preparations were analyzed for hexose by the method of Radin et al. (17), in the form of galactose equivalents. Sphingosine and sialic acid (N-acetyl neuraminic acid) were measured by the procedures of Yamamoto and Rouser (18) and Jourdian et al. (19), respectively.

Purification protocol. The C/M extract of the acetone powder was first separated on a silicic acid column. The cerebroside-containing fractions, determined by qualitative TLC, were then separated on preparative thin layer plates, and the cerebroside fractions were eluted. The 50% C/M fractions (containing essentially all of the gangliosides) were subjected to mild alkaline methanolysis and separated by ion-exchange HPLC. HPLC fractions, with the exceptions of GT_x and the second peak of GD_3, were finally purified by TLC and eluted. GLC analysis was performed on the purest preparation of each molecule.

RESULTS

Fresh-frozen bovine pineals averaged 168.5 mg. Three hundred pineals yielded a total of 10.42 g of acetone powder, a loss of about 80% of total weight. The combined, dried C/M extracts of three different acetone powders represented 2.4 ± 0.3% of the fresh tissue weight, or 12 ± 1.5% of the weight of the acetone powders.

Neutral glycosphingolipids. The C/M extract is highly enriched in glycolipids, and especially in sphingolipids, although some phospholipids remain. The fractions that are eluted from the silica column in A/C (1:1) and A/C (3:1) contain relatively nonpolar lipids, the major one of which then chromatographed in thin layer solvent A indistinguishably from purchased standards of cerebroside. It gave two carbohydrate-positive spots, R_f 0.70 and 0.78, when commercial cerebroside on the same plate gave spots at 0.69 and 0.77. All of the 0.78 R_f material was eluted from the silica column by A/C 1:1. The 0.70 R_f material was spread between both fractions. Methanolysis and GLC analysis of material from both bands yielded only galactose, fatty acids and peaks with the relative retention times of sphingosine derivatives. These were not sufficiently reproducible to quantify. The ratio of galactose to total fatty acid was 1.01 for the combined, eluted material from the two bands. The fatty acids of the more polar band could only be quantified after trimethylsilylation, which may be because of the presence of 2-hydroxy fatty acids. The combined bands, identified as galactocerebroside, are present in a quantity of 1.45 μmol/g fresh tissue. This represents 30.2% of the total lipid-bound hexose in the C/M extract. This level is about one-third of the usual quantity for cerebroside in mammalian central nervous system gray matter, and about 3% of that for white matter. While this figure for cerebroside content must be regarded as an approximate minimum, it has previously been reported that cerebroside was not present in bovine pineal (20). We would anticipate that procedural losses were greater for cerebrosides than for

gangliosides, as significant amounts of neutral lipid might be expected to dissolve in the original acetone extraction. Losses of standards during silicic acid chromatography average less than 15%.

The major gangliosides. All of the sialic acid-containing material in the extract was eluted from the silicic acid column by C/M, 1:1. This fraction was further purified, after mild alkaline methanolysis and dialysis, by ion-exchange HPLC (14). Because of overlap between gangliosides with a similar number of sialic acid residues, especially among monosialogangliosides, early peaks from the chromatogram were further purified by TLC. Each band, eluted from TLC plates after chromatography in solvent A, was methanolyzed, and the resulting methyl glycosides and fatty acid methyl esters were identified and quantified by GLC, as described in Methods. Table 1 shows the component ratios, as determined by methanolysis and GLC, of the major gangliosides of pineal. The ratios were determined with the use of correction factors in the manner described in Methods. The names given to each are partially based on these data. Table 2 shows the TLC R_f in solvent A, the position(s) of elution in HPLC and the relative amounts in fresh pineal of each of the five detectable gangliosides, compared to the behavior of purchased standards in the same systems. Peaks eluting from HPLC prior to 0.35 M NaCl require further purification on TLC, as there is significant overlap. The early part of the elution is also the portion of the chromatogram in which those contaminants destroyed by alkaline methanolysis appear. The portion of the chromatogram eluting after 0.35 M NaCl, containing the second peak of GD_3 and all of GT_x, appears to provide purified gangliosides directly, even without the alkaline treatment. GD_3 has been observed to separate into two different peaks in other chromatographic systems (8), possibly due to variation in its hydrophobic moiety.

These ganglioside identifications are based on the chromatographic characteristics and gas chromatographically determined component ratios of each extracted molecule. More definitive identification will have to await precise physical analysis, by techniques such as fast atom bombardment mass spectroscopy.

GD_3 and GM_3 combined accounted for 82.8% of the total ganglioside of the tissue, with GD_3 alone present

TABLE 1

Component Ratios of the Five Major Gangliosides of Bovine Pineal[a]

Ganglioside[b]	N-Acetyl neuraminic acid	N-acetyl galactosamine	Galactose	Glucose	Total fatty acid
GD_3	2.01 ± 0.16	<0.01	1.02 ± 0.13	1.0	0.92 ± 0.19
GM_3	0.97 ± 0.06	<0.01	0.94 ± 0.05	1.0	0.77 ± 0.10
GD_{1a}	1.80 ± 0.004	0.88 ± 0.09	1.82 ± 0.03	1.0	0.76 ± 0.12
GM_1	1.08 ± 0.19	0.64 ± 0.08	1.85 ± 0.06	1.0	1.07 ± 0.45
GT_x	2.99 ± 0.33	0.72 ± 0.49	1.05 ± 0.01	2.0	N.D.

[a]Determined as described in Methods. Normalized to glucose. N.D., not determined. Each number is the average of at least three separate determinations.

[b]Tentative assignments—see text.

TABLE 2

Comparative Chromatographic Characteristics of the Major Gangliosides of Bovine Pineal

Pineal ganglioside[a]	Standard ganglioside	R_f in solvent A[b]	M NaCl for peak(s) elution[b]	Amount (nmol/g fresh tissue)
GM_3		0.39	0.095 ± 0.019 0.154 ± 0.025	100
GM_1		0.19	0.095 ± 0.019 0.154 ± 0.025	40
	GM_1	0.18	0.108 ± 0.004	
GD_3		0.21	0.271 ± 0.033 0.382 ± 0.016	300
GD_{1a}		0.13	0.276 ± 0.026	40
	GD_{1a}	0.12	0.268 ± 0.027	
	GD_{1b}	0.05	0.386 ± 0.002	
GT_x		N.D.	0.462 ± 0.005	3
	GT_{1b}	0.05	0.536 ± 0.01	

[a]Tentative assignments—see text.

[b]Average of at least three separate runs.

Extracted and standard gangliosides on same TLC plate. N.D., not determined.

in pineal at 4.4 times the concentration found in normal gray matter (21). GM_1 and GD_{1a}, the major gangliosides of whole brain, are present only at about 1/8 of their whole brain level (21). The fatty acid profiles of the two lactosyl gangliosides were similar to each other and different from those of the gangliotetraose molecules GD_{1a} and GM_1. Table 3 gives the average values for all those fatty acid methyl esters present in >1% of the total. The pineal lactosyl gangliosides are characterized by significantly less stearate and significantly more long chain saturated fatty acids (C20, C22 and C24) than are usually found in brain gangliosides (22). This suggests the possibility of a metabolic relationship between the two lactosyl gangliosides, but no data about their metabolism in pineal are available.

GT_x. The ganglioside in fifth largest quantity in bovine pineal, designated GT_x in the tables, was reproducibly isolated from the freshly prepared acetone powder, but only in amounts of 0.62% of the total ganglioside. The carbohydrate composition was reproducible from three separate isolations and purifications, but a large enough quantity of material for total characterization will have to await a significantly larger extraction. On the basis of its chromatographic behavior, solvent extraction characteristics and carbohydrate content, it is reasonable to conclude that the molecule is a novel trisialoganglioside, having a glucose/galactose ratio of 2:1, in addition to the three sialic acid residues and a single amino sugar. It elutes from ion-exchange HPLC earlier than a GT_{1b} standard, but later than any standard disialoganglioside.

The sum of the five detectable gangliosides comes to 0.48 μmol/g fresh tissue. This represents about 1/4 (24.2%) of the level of total gangliosides of mammalian gray matter and about 60% of that of mammalian white matter (21). In our work, measurements of total sphingosine (18) and total sialic acid (19) in the crude C/M extract consistently gave figures less than that of the combined, isolated glycosphingolipids. The combined total anthrone-positive hexose of the isolated gangliosides represents about 27% of the total hexose in the C/M extract. Therefore, all five gangliosides combined with both cerebrosides represent a 57 ± 6% recovery of total measurable hexose from the C/M extract. Losses during chromatography undoubtedly account for a portion of the remainder. In addition, bovine pineals have a small quantity of non-sphingosine-containing glycolipids (23). However, the combined yield of lipid-bound sialic acid reported here (0.829 μmol/g fresh tissue) is more than fourfold higher than that previously reported (24) for bovine pineals, and both the pattern and yield are reproducible.

TABLE 3

Fatty Acid Profiles of Four Major Gangliosides of Bovine Pineal, in Mole %[a]

Fatty acid	Lactosyl gangliosides	Gangliotetraose gangliosides
16:0	3.5 ± 4.9	2.5 ± 3.5
16:1	2.5 ± 0.71	7.0 ± 0.001
18:0	56.5 ± 7.78	82.0 ± 2.83
18:1		8.5 ± 0.71
20:0	18.0 ± 2.83	
22:0	13.0 ± 2.83	
24:0	6.5 ± 2.12	

[a]Only those found in as much as 1% of the total.

DISCUSSION

GD_3 has previously been shown, in the rat's central nervous system, to be most highly concentrated in the locus coeruleus (25). The locus coeruleus has the largest number of noradrenaline-containing neurons in the brain, and it also contains many noradrenergic receptors, in feedback collateral synapses (26). In vitro studies have, in the past, led some investigators to hypothesize that GD_3 was specifically involved in the action of neurotransmitters (27,28). While our data combined with the above evidence might indicate a possible correlation between GD_3 and noradrenaline, other data indicate that that may not be the case. GD_3 is also the predominant ganglioside in whole retina (29) and in rod outer segments (ROS) (30). ROS presumably contain no synapses or transmitters at all, and noradrenaline is not a significant presence in retina. In addition, many investigators have found GD_3 to be the predominant ganglioside in some tumor cells (31) and in some embryonic nervous tissue (32). These observations have prompted the hypothesis that a high concentration of GD_3 is characteristic of undifferentiated cells. Further, Seyfried and Yu (8) have shown that GD_3 is the major ganglioside of some glia. Pineal bodies contain glia, noradrenergic synapses and intracellular membrane structures somewhat similar in appearance to rod discs (33). The GD_3 present in pineal could be associated with any one or more of these structures. Without specific localization studies, the question of anatomical location of GD_3 in pineal cannot be answered. It is clear that GD_3 is present in diverse locations and may have diverse functions.

Dreyfus et al. (34) demonstrated that neuraminidase is active in ROS, with greatest activity expressed on GD_3. GM_3, the product of the removal of a single sialic acid from GD_3, was also a good substrate. This evidence places these two gangliosides in a separate metabolic pool from the other known gangliosides of ROS, for most of the others give the same product (GM_1) from neuraminidase activity, and are not catabolized to non-gangliosides in situ. The fact that GD_3 and GM_3 share a similar fatty acid profile in pineals, which is different from that of the other gangliosides, may also reflect their being in a separate metabolic pool and may represent a further similarity between the two tissues. The evidence from both tissues for a precursor-product relationship between gangliosides is in contrast to the usual situation in neurons, where a complete pattern of gangliosides is synthesized, and later catabolized, in the cell body. There is usually no metabolic relationship among gangliosides in their functional membrane locations (7).

Pineal and retina have similarities other than ganglioside content and metabolism. Both tissues are light responsive, although the response is indirect for the pineal (4). Both tissues are high in cGMP (35), and they both synthesize melatonin, the pineal hormone (36). In any case, clarification of the anatomical localization of GD_3 and GM_3 in pineal will be a meaningful advance in pineal physiology.

Of the glycosphingolipids of pineal, that which appears to be most characteristic of the tissue, based on these studies, is GT_x. While the data we have presented about

it are incomplete for the hydrophobic moiety, its chromatographic behavior and carbohydrate content would indicate that it is a trisialoganglioside. The content of glucose is twice that of galactose, which is unusual for mammalian gangliosides, but has been previously reported for embryonic chicken brain (37). A more complete determination of the detailed structure of GT_x would be interesting. If GT_x is in fact characteristic of pineal, its localization in the tissue would also be significant and possibly instructive about function.

It is reasonable to expect at least one of the pineal gangliosides to be concentrated in, and associated with, the noradrenergic synapses which innervate it. GD_3 and GM_3 appear to be either associated with glial cells (8) or localized intracellularly (30) in other studies. GM_1 and GD_{1a} may be associated with synapses, but their prominence in whole brain would imply that they are not specifically associated with noradrenaline. Therefore, GT_x may be the most likely to have a specific association with pineal synapses. The clarification of this interesting possibility will have to await future histological studies of its localization.

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Onset and Persistence of Changes in Intestinal Transport Following Dietary Fat Manipulation

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In this study we determined the time-course for the onset and the loss of the effect of short-term feeding rats isocaloric semisynthetic diets containing a high content of saturated (HS) or polyunsaturated (HP) fatty acids on the jejunal and ileal uptake of medium- and long chain fatty acids, cholesterol and glucose. Animals were fed HP or HS for 3, 7 or 14 days; then the diet was switched to standard Purina® rat chow for a further 3, 7 or 14 days. The uptake of medium chain fatty acids was unchanged. The differences between HP and HS in glucose uptake occurred within 3 days, but persisted for 14 days, whereas there were qualitative as well as quantitative changes in the pattern of lipid uptake: differences in uptake of stearic, oleic, linoleic and linolenic acids and cholesterol occurred after 7 days of feeding HP or HS. Jejunal uptake of linoleic acid was greater in HP than HS on day 7, but HS was greater than HP on day 14. The effect of diet on lipid uptake was similar in the jejunum and ileum. The altered uptake of stearic and oleic acids persisted after the rats were switched back to chow, whereas the uptake of the other nutrients became similar. Thus, (i) changes in dietary content of saturated and polyunsaturated fatty acids have early effects on intestinal transport function; (ii) some of these changes persist even when animals are returned to feeding on chow; and (iii) glucose transport is rapidly altered by dietary changes, whereas lipid uptake changes only after 7 days. We conclude that the transport function of the intestine is responsive to changes in dietary fatty acids.
Lipids 22, 22–27 (1987).

The passive permeability properties of intestine are altered in response to manipulations in the dietary content of cholesterol, fatty acid, carbohydrate, protein and essential fatty acids (1–4). Manipulation of dietary lipid composition also influences the jejunal adaptive response following ileal resection in rabbits (5). Feeding rats a semisynthetic isocaloric diet containing a high or a low ratio of saturated-to-polyunsaturated fatty acids alters jejunal active transport of glucose and leucine as well as passive uptake of fatty acids, cholesterol and bile acids (6). Dietary alteration in the amount of the macronutrients alters jejunal morphology as well as jejunal brush border membrane lipid composition in control and diabetic rats (7). Substitution of polyunsaturated fatty acids for saturated fatty acids has no significant effect on brush border membrane content of cholesterol or phospholipids (6). On the other hand, Brasitus and coworkers (8) reported that modification in the saturation of dietary fatty acids is associated with changes in the fatty acyl content of intestinal membrane phospholipids; we have reported similar findings of changes in brush border membrane phospholipid fatty acids after two weeks' feeding with the diets used in this study (9). The present study extends these observations by indicating the rapid onset of these transport changes and their persistence when the diets are stopped.

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METHODS

Animals and diets. Female Wistar rats (40–56 days old), 220–250 g, were used. Principles for the care and use of laboratory animals approved by the Canadian Federation of Biological Societies and the Council of the American Physiological Society were observed. Animals were allowed access to water and food ad libitum until the morning of the study. They were fed one of three diets for 3, 7 or 14 days: standard Purina® rat chow or a semipurified diet with 20% (w/w) fat of either a high or low polyunsaturated-to-saturated fatty acid (P/S) ratio (6). The semipurified diets were nutritionally adequate, providing all known essential nutritional requirements. The diet high in polyunsaturated fatty acids (HP) provided ca. 22% of calories and 55% of total fatty acids (% w/w) as 18:2ω6 (linoleic acid), whereas the diet high in saturated fatty acids (HS) provided ca. 2.2% of calories and 5% of total fatty acids as 18:2ω6. The percentages of the total fatty acids were approximately as follows: for 16:0, chow 16%, HP 10% and HS 21%; for 18:0, chow 5%, HP 2% and HS 34%; for 18:1, chow 24%, HP 22% and HS 32%; for 18:2, chow 41%, HP 55% and HS 5%; and for 18:3, chow 4%, HP 5% and HS 1%. Other groups of rats fed either the HP or the HS fatty acid diet for two weeks were then switched back to chow for 3, 7 or 14 days.

Animals were weighed at the beginning of the study and about twice weekly thereafter. Intestinal weights were determined at the time of the absorption studies.

Probe and marker compounds. [^{3}H]Inulin (mol wt ∿5,000) as supplied by the manufacturer (New England Nuclear Corp., Boston, Massachusetts) was used in each experiment. [^{14}C]Labeled probes included hexanoic acid (6:0), octanoic acid (8:0), decanoic acid (10:0), lauric acid (12:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3), cholesterol and D-glucose. Unlabeled and [^{14}C]labeled probes were supplied by Sigma Co. (St. Louis, Missouri) and by New England Nuclear, respectively. Probes were shown to be more than 99% pure by high performance liquid chromatography.

Tissue preparation. Animals were anesthetized by intraperitoneal injection of sodium thiopentol. A 15-cm length of proximal jejunum or distal ileum was rapidly removed and gently rinsed with 50 ml of cold saline, as described in detail elsewhere (10,11). The intestine was opened along its mesenteric border and the mucosal surface was carefully washed with cold saline to remove visible mucus and debris. Pieces of intestine were cut from the segment, mounted as flat discs in the incubation chambers and placed in preincubation beakers. Preincubation beakers contained oxygenated Krebs-bicarbonate buffer (pH 7.4) at 37 C. Tissue discs were preincubated for 10 min to allow for equilibration at this temperature.

The transport chambers were then transferred to other incubation beakers for specific experiments. Preincubation and incubation solutions were mixed at identical stirring rates with circular magnetic bars, and the stirring rates were precisely adjusted by means of a strobe light. Stirring rates were reported as revolutions/min (rpm) at which the stirring bar was driven. The stirring rate of 600 rpm was selected to achieve low effective resistance of the intestinal unstirred water layer (11).

Determination of uptake rates. After preincubation, chambers were transferred to other beakers containing [^{3}H]inulin and various [^{14}C]probe molecules in oxygenated Krebs-bicarbonate buffer. After incubation of intestinal discs in labeled solutions for 6 min, each experiment was terminated by removing the chamber and quickly rinsing the tissue in cold saline for ca. 5 sec. The exposed mucosal tissue was then cut out of the chamber with a circular steel punch and gently blotted on filter paper. Tissue was dried overnight in an oven at 75 C. The dry weight of this tissue was determined, samples were saponified with 0.75 N NaOH, scintillation fluid was added and radioactivity was determined by means of an external standardization technique to correct for variable quenching of the two isotopes (11).

Individual experiments. Micellar solutions of the long chain fatty acids (18:0, 18:1, 18:2 and 18:3) and cholesterol were prepared (12) by solubilizing these lipids in 20 mM taurodeoxycholic acid (TDC). The concentrations of solutes were: cholesterol, 0.05 mM in 20 mM TDC; stearic acid, 0.1 mM; and long chain unsaturated fatty acids (18:1, 18:2 and 18:3), 1.0 mM in 20 mM TDC. Glucose-containing solutions (1 mM and 40 mM) were prepared in Krebs-bicarbonate buffer without TDC. These concentrations of glucose were selected to represent values at (1 mM) and above (40 mM) the Michaelis constant for D-glucose uptake into rat jejunum (13). A change in uptake of 1 mM glucose would be achieved by alteration in the value of the apparent Michaelis constant, whereas a change in uptake of 40 mM glucose would be achieved by alteration in the value for maximal transport rate and/or passive permeability coefficient (14).

Dietary changes. One group of animals was fed Purina® rat chow throughout the study, one group was fed the semisynthetic high polyunsaturated fatty acid diet (P) for 3, 7 or 14 days, and one group was fed the high saturated fatty acid diet (S) for 3, 7 or 14 days. A subgroup of animals was fed S or P for 14 days and was then switched back to Purina® rat chow for a further 3, 7 or 14 days.

Morphology. Morphometric measurements were completed on 1-cm sections of jejunum and ileum fixed in Bouin's solution, dehydrated, embedded in paraffin wax, sectioned for light microscopy and stained with H & E using standard techniques. The mucosal surface area was determined as reported previously (7,15,16).

Expression of results. The rate of uptake of solutes was calculated after correcting the total tissue [^{14}C]radioactivity for the mass of the solute molecule present in adherent mucosal fluid. Uptake rates were expressed as nmol of the solute molecule taken up into the mucosa per 100 mg dry weight of tissue/min (nmol/100 mg/min). Values obtained from different dietary groups are reported as the mean ± SEM of results observed for a minimum of six animals in each group.

The student's t-test was used to test the significance of the difference between the means of animals fed HP vs HS. Analysis of variance (ANOVA) demonstrated that there were no significant differences in means among 3,7 and 14 days off HP or HS, and therefore the values for 3, 7 or 14 days off a given diet were combined to provide one mean for HP-off and one mean for HS-off.

RESULTS

Animal characteristics. Body weight gain on days 3, 14, 21 and 28 was similar for animals fed the high polyunsaturated (HP) or high saturated (HS) diet or chow (Fig. 1A). In jejunum and ileum of P, the weight of the tissue declined between days 3 and 7, but remained constant from day 7 to 28. For S, the weight of the ileal tissue remained constant, whereas the weight of the jejunum fluctuated. The jejunal mucosal surface area (MSA) of animals fed HP increased above control or HS values on day 3, but at all other times on or off HP or HS, there was no diet-associated effect on jejunal or ileal mucosal surface area (Fig. 2).

Uptake of glucose. Feeding HP or HS had no effect on the jejunal or ileal uptake of 1 mM glucose, except for greater uptake in HP than HS after 3 days (Fig. 3).

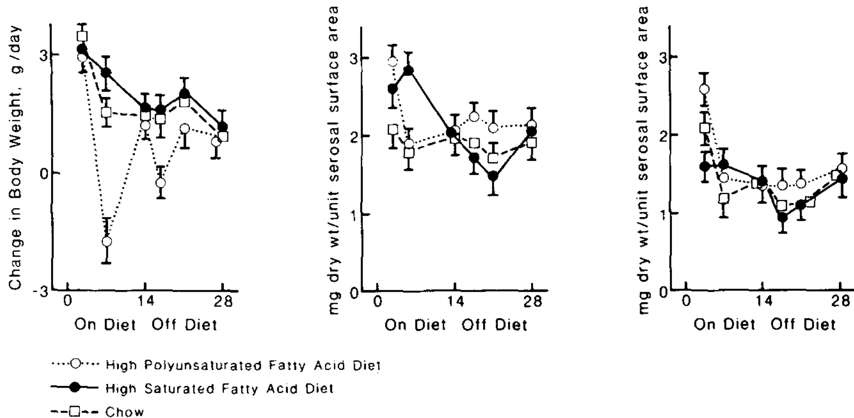

FIG. 1. Characteristics of rats while on or off the high polyunsaturated or high saturated fatty acid diets. (A) Body weight; (B) jejunal weight; (C) ileal weight. Mean ± SEM of the results of 6–9 animals.

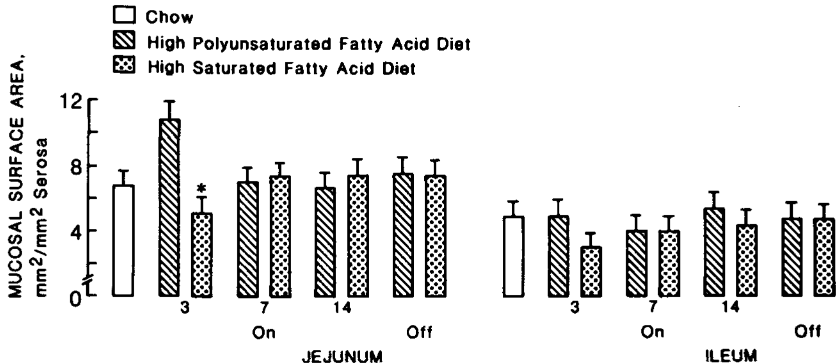

FIG. 2. Effect of feeding the high polyunsaturated or high saturated fatty acid diets on the jejunal and ileal mucosal surface area. Mean ± SEM of the results of 6–9 animals. An asterisk indicates a significant difference between the animals fed the high polyunsaturated fatty acid diet vs chow or the high saturated fatty acid diet and chow.

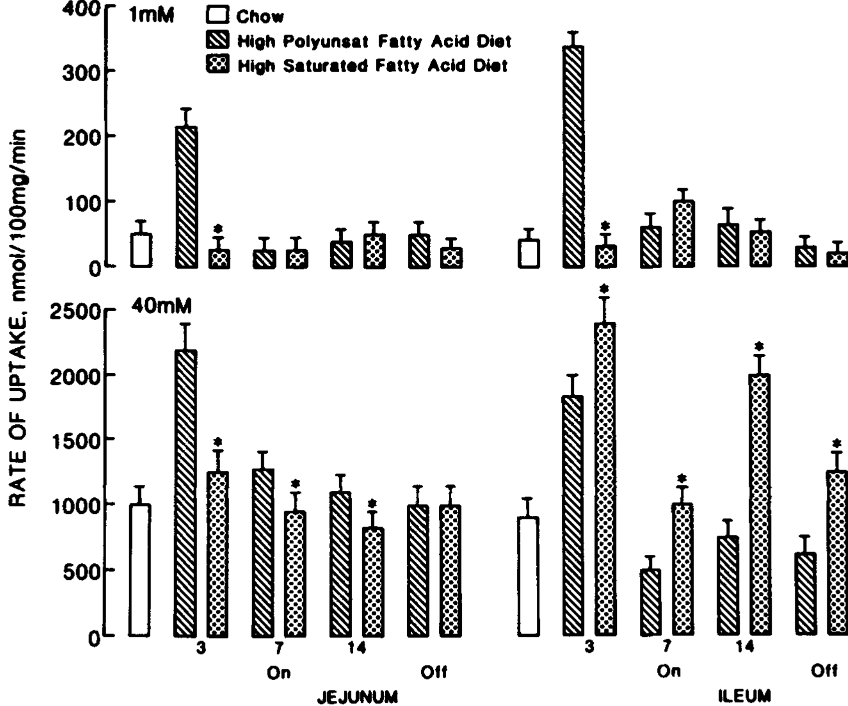

FIG. 3. Effect of feeding the high polyunsaturated or high saturated fatty acid diets on the jejunal and ileal uptake of 1 mM and 40 mM glucose. Mean ± SEM of the results of 6–9 animals. An asterisk indicates a significant difference between the animals fed the high polyunsaturated fatty acid diet vs chow or the high saturated fatty acid diet and chow.

Jejunal uptake of 40 mM glucose was also greater in HP than HS on day 3, 7 or 14, whereas in the ileum uptake was greater in HS than HP, and this difference persisted when the rats were switched back to chow.

Lipids. The uptake of hexanoic, octanoic and decanoic acids was similar at all times in HP and HS, whereas jejunal and ileal uptake of lauric acid was greater in HP than in HS at days 3 and 7 (not shown). The jejunal uptake of stearic acid was greater in HP than HS at day 7, but lower in HP than HS at day 14 (Fig. 4); in the ileum uptake was greater for HP than HS at both days 7 and 14. The jejunal uptake of oleic acid was similar in HP and HS, although ileal uptake was greater for HP than HS at days 3 and 7. Linoleic acid uptake into the jejunum was higher in HP than HS at day 3; no differences were noted with larger periods of feeding. Finally, cholesterol uptake was greater in HS than in HP only at day 14. When the animals were switched from HP or HS to chow,

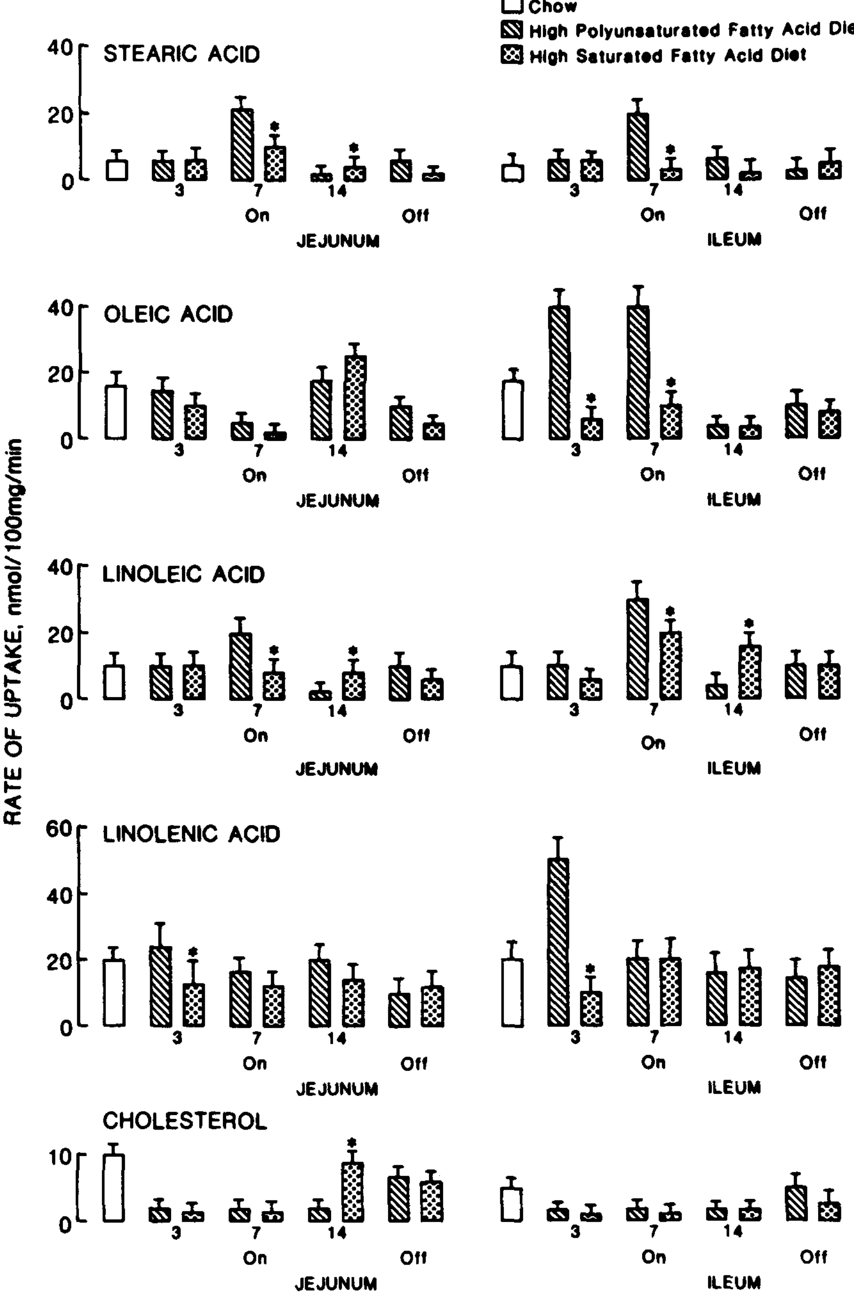

FIG. 4. Effect of feeding the high polyunsaturated or high saturated fatty acid diets on the jejunal and ileal uptake of fatty acids and cholesterol. Mean ± SEM of the results of 6–9 animals. An asterisk indicates a significant difference between the animals fed the high polyunsaturated fatty acid diet vs chow or the high saturated fatty acid diet and chow.

uptake of cholesterol, linolenic and linoleic acids became similar, but uptake of oleic and stearic acid remained lower in animals previously fed HS than HP.

DISCUSSION

An enhanced awareness of the importance of the effect of dietary manipulation on intestinal transport function (17) arose from studies of intestinal adaptation following intestinal resection (5,18). Starvation or semistarvation alters intestinal transport function (19), but the specific nutrient composition of the diet is also important in modulating the form and function of the intestine (1–4). Changes in intestinal uptake of lipids occur under a variety of experimental conditions, such as aging, diabetes, ileal resection, chronic ethanol exposure, and following abdominal irradiation (20). These changes cannot be fully explained by alterations in the effective resistance of the unstirred water layer (21), but may be associated with changes in the "fluidity" of the intestinal brush border

membrane (7,22) and with an alteration in the quantity or type of brush border membrane phospholipids (16, 23–26). Modifications in the dietary content of carbohydrate, cholesterol, essential fatty acids or protein influence intestinal transport function in both control and diabetic rats (3,4), and these dietary manipulations are associated with changes in the phospholipid content of the brush border membrane (7). For these reasons, it seems likely that the brush border membrane phospholipids play an important role in the modulation of intestinal transport. However, changes in the P/S ratio in the diet of rats also altered intestinal uptake of lipids, hexoses and leucine without associated changes in villus morphology or brush border membrane phospholipids (6).

A recent report has suggested that variations in the saturation of dietary fatty acids will give rise to changes in fluidity of brush border membranes and to changes in the fatty acyl content of membrane phospholipids (9,21). Before making further attempts to define the relationship between brush border membrane phospholipids, fatty acids and nutrient uptake, we have explored the time-course of changes in intestinal uptake of nutrients by feeding semisynthetic diets high in saturated or polyunsaturated fatty acids for variable periods. Based on the previous study, which demonstrated alterations in uptake of lipids, glucose and leucine 14 days after feeding mature rats the semisynthetic diets high in HS or HP fatty acids (6), it was anticipated that shorter periods of feeding HS or HP would be associated with qualitatively similar findings. The effect of feeding HS or HP for 14 days on uptake of 40 mM glucose, stearic and linoleic acids and cholesterol was confirmed (Figs. 3 and 4). However, in no instance were the changes progressive: the lower uptake of 1 mM glucose in HS than HP occurred only on day 3, and the difference in jejunal uptake of 40 mM glucose was greater on day 3 than on day 14 (Fig. 3). In addition, the qualitative nature of the changes varied with time; for example, uptake of stearic and oleic acids was greater in HP than HS on day 7, but greater in HS than HP on day 14. Furthermore, although glucose uptake changes on day 3, lipid uptake changed only after 7 days of feeding HS or HP. This suggests that a different mechanism was responsible for the rapid alterations in glucose uptake but slower alterations in lipid uptake in response to diet. Changing dietary saturation of fatty acids does not result in perturbations of brush border membrane cholesterol or phospholipids (6), although feeding HP and HS results in alterations in membrane acyl components (unpublished observations; 8). It is uncertain which portion(s) of the membrane are affected, but previous authors have suggested that there may be discrete microdomains within the lipid membrane involved in the binding of different proteins (27,28). This possibility has previously been raised on the basis of theoretical studies (29). In addition, the major portion of intestinal uptake of palmitic acid occurs from the upper third of the villus (30). It is possible that the diet-associated changes in lipid uptake may be mediated by another domain, such as the bulk phase lipids in the membrane.

Feeding a diet enriched in linoleic acid increases the proportion of membrane lipid comprised of this fatty acid (8) and increases its uptake on day 7 but not day 14. However, dietary supplementation with linolenic acid did not alter the uptake of this fatty acid. Thus, an alteration of a given lipid in the membrane does not necessarily change the uptake of that lipid. Furthermore, the uptake of cholesterol is increased in HS compared with HP (Fig. 4), even though the membrane cholesterol is unchanged (6). It is likely that alterations in lipid uptake are due to complex metabolic alterations that change the "fluidity" of the membrane and thereby alter passive permeability properties and lipid uptake rates.

The time-course of changes in uptake varied between the jejunum and ileum (Figs. 3 and 4). It is unknown how far along the intestine the dietary challenge extends, but presumably the magnitude of the challenge would diminish with passage along the intestine. However, the microsomal membrane lipids are qualitatively different in the jejunum than in the ileum (unpublished observations). Furthermore, the hydroxymethylglutaryl CoA reductase activity is different in the proximal as compared with the distal intestine (31,32). Thus, the different transport response of the ileum and the jejunum to dietary changes may be due to more than a gradient of the dietary load along the length of the intestine. While manipulation of dietary fatty acids might be predicted to have an influence on lipid uptake (since qualitatively similar changes were noted along the length of the intestine) (Fig. 4), the greater uptake of 40 mM glucose into the ileum of HS than HP might counteract the lower jejunal uptake (Fig. 3). Thus, any predictions must be made cautiously about the potential role of feeding a saturated fatty acid diet to alter blood glucose concentrations after a meal.

Since the mechanism of the altered intestinal uptake in response to dietary changes has not yet been established, it is not possible for us to indicate the explanation for the persistent difference between HS and HP in the uptake of stearic and oleic acid (Fig. 4). None of the alterations in uptake could be explained by differences in the animal's body weight, intestinal weight, or mucosal surface area (Fig. 2). Nonetheless, it is important to determine for just how long there is a late effect of dietary lipids on intestinal transport function.

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Intestinal Cholesterol and Oleic Acid Uptake from Solutions Supersaturated With Lipids

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To test the role of nonmicellar phases in lipid absorption, intestinal uptake of fatty acids and cholesterol has been studied in vitro from supersaturated and micellar solutions. The micellar solubility limit at equilibrium was established for cholesterol and oleate/monoolein (2:1) at pH 6.7 with 10 mM taurocholate. Uptake by rat intestinal everted sacs was measured during incubation of 5 min. Cholesterol uptake increased linearly with the cholesterol content of micellar or supersaturated solutions up to a supersaturation of 150%. Oleate uptake, by contrast, remained essentially the same from either saturated or supersaturated (130–280%) mixtures. The difference between cholesterol and oleate uptake rates is explained by their distinct effects on micellar size, which is unchanged by cholesterol supersaturation but is increased by oleate. Solutions largely supersaturated (280%) with oleate-monoolein are polydisperse and contain viscous isotropic and paracrystalline phases similar to those observed during lipid absorption. These results suggest that, in the presence of such solutions, uptake occurs from both the micellar saturated and nonmicellar supersaturated phases.
Lipids 22, 28–32 (1987).

Lipid absorption is thought to occur from a micellar phase resulting from fatty acids and monoglyceride solubilization by bile salts in the intestinal lumen. Micelle formation could be necessary to overcome the resistance of the unstirred water layer so that lipolysis products reach the epithelial brush border. More recently it was shown that during fat digestion this micellar phase is probably mixed with a lamellar liquid crystalline phase and a viscous isotropic phase (1,2). These two phases are partially soluble in water and cannot be clearly separated from the micellar phase by centrifugation. It has been suggested that they could be substrates from which fat absorption occurs. The aim of this work was to test this hypothesis by measuring intestinal uptake of lipids from saturated micellar solutions and from largely supersaturated solutions so that they contain micelles in equilibrium with nonmicellar phases.

MATERIALS AND METHODS

Chemicals. Sodium taurocholate was purchased from Calbiochem (La Jolla, California). Sodium oleate, monoolein and cholesterol, 99% pure, were obtained from Sigma Chemical Co. (St. Louis, Missouri). Ninety percent of the monoolein was the α-isomer and the balance was the β-isomer. [4-^{14}C]Cholesterol (40–50 mCi/mmol) and [1-^{14}C]oleic acid (45–55 mCi/mmol) were purchased from CEA-France (Saclay, France) and were found to be greater than 98–99% pure. [^{3}H]Inulin was obtained from Amersham-France SA (Les Ulis, France), 97% pure.

Preparation of mixed micelles. Solutions of lipids in 10 mM taurocholate were prepared by coprecipitation (3). The solubility limits were determined at pH 6.7 in phosphate buffer of 0.15 M Na^+ at 37 C after an equilibration time of 7 days. In a first series of experiments, increasing amounts of oleate/monoolein (molar ratio 2:1) were added to a constant taurocholate concentration of 10 mM. In a second series, various amounts of cholesterol were added to taurocholate/oleate + monoolein = 55:45 mixtures with oleate/monoolein = 2 (molar ratios). The different solutions used in the study are plotted (Fig. 1) in rectangular coordinates.

The separation of a second phase (cholesterol crystals, paracrystalline phases) from the isotropic mixtures was monitored by polarizing microscopy and the Tyndall phenomenon following classical criteria (1,4).

Molecular weight determination. These assays were performed at 25 C with the aid of a Spinco-Beckman model-E analytical centrifuge equipped with temperature and speed controls (72 min at 60,000 g). The homogeneity of the micellar solution was tested by the sedimentation coefficient determination, for which a double sector capillary cell with synthetic boundary and a schlieren optical system was employed. A phosphate buffer was used (10 mM, pH 6.7, 0.15 M Na^+). For micellar weights, the Yphantis method (5) was used and the partial specific volume of mixed micelles was experimentally measured in a Parr microdensimeter (6); this value was used for calculating the weight of the micelles.

Cholesterol uptake. The preparation of everted sacs has been previously described (7). The small intestine of male Wistar rats (250–280 g) was removed and rinsed with cold saline and immediately everted over a glass rod. A

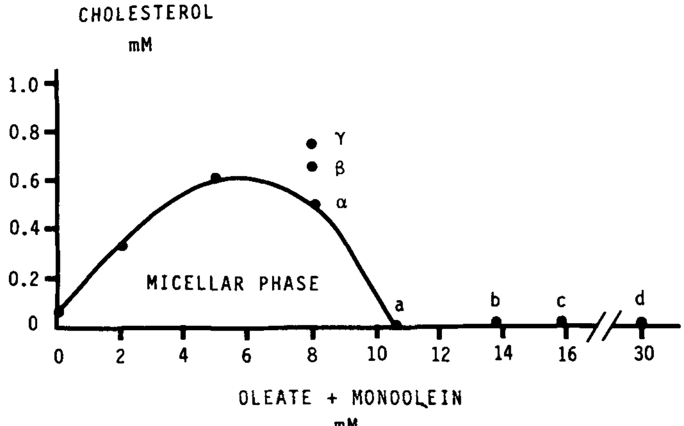

FIG. 1. Composition of the different test solutions in rectangular coordinates. Lipid solubility limits were determined for a constant taurocholate concentration (10 mM). Cholesterol saturated and supersaturated solutions are indicated as α, β and γ (100, 130 and 150% saturation, respectively). Oleate + Monoolein saturated and supersaturated mixtures are indicated as a, b, c and d (100, 130, 150 and 280% saturation, respectively). This diagram does not allow the determination of the physical structures of the test solutions, as they were used before complete equilibrium was obtained. From microscopic examination, all of the solutions except d were homogeneous and behaved like supersaturated micelles.

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10–15 cm segment distal to the ligament of Treitz was used in this experiment. Sacs 1.5-cm long from the segment were tied off sequentially and kept in cold buffer solution until used. Incubation was done immediately at 37 C for 5 min and at a stirring rate of 750 rev min^{-1} in micellar solutions that contained [^{14}C]cholesterol or [^{14}C]-oleic acid and trace amounts of [^{3}H]inulin as a radiolabeled volume marker. Following incubation, the sacs were removed, rinsed in cold saline and dried overnight at 60 C. This temperature did not lead to any loss of oleic acid. They were then weighed and solubilized with Soluene-350 and Dimilume-30 (Packard Instruments Co., Downers Grove, Illinois), and the liquid scintillation counting was carried out using Tri-Carb 300C counter (Packard Instruments Co.). The kinetics of cholesterol or oleate uptake were measured between 3 and 15 min. A linear relationship existed between the amount of cholesterol or oleate uptake and time. Therefore, a 5-min incubation time was chosen because 5 min was sufficient for the unstirred water layer to become uniformly labeled with the marker, but not so long as to damage the membrane. The data were expressed as nmoles of cholesterol or oleate 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.

TABLE 1

Effect of Oleate or Cholesterol Supersaturation on Apparent Molecular Weight of Mixed Micelles of Taurocholate/Oleate/Monoolein With or Without Cholesterol

Supersaturation (%)	V[a]	S[b]	aMW[c] (daltons)
Oleate			
100	0.890	0.90	45,000
130	0.936	0.80	60,000
150	0.964	0.63	115,000
Cholesterol			
100	0.880	1.00	40,000
130	0.880	0.92	40,000
150	0.880	1.00	40,000

[a]Partial specific volume.
[b]Sedimentation coefficient.
[c]Apparent molecular weight.

RESULTS

Solubility limit. The cholesterol solubility limit in micellar form was determined at pH 6.7 for a taurocholate/oleate + monoolein = 55:45 (oleate/monoolein = 2:1) molar ratio. The cholesterol solubility limit was 0.49 mM. From these data, we prepared saturated and supersaturated solutions (130% and 150% saturation with cholesterol). The mixtures were used for uptake studies within 24 hr following preparation so that the supersaturated solutions did not contain cholesterol crystals and were optically clear (Tyndall−). The oleate/monoolein solubility limit (oleate/monoolein = 2:1) in 10 mM taurocholate was estimated to be 10.55 mM.

Molecular weight. For saturated and supersaturated solutions, we determined the partial specific volume, the sedimentation coefficient and the apparent molecular weight. As shown in Table 1, the results were different for oleate supersaturation and for cholesterol supersaturation. When oleate concentration increased from 10.55 mM to 15.80 mM, the apparent molecular weight increased significantly from 45,000 to 115,000. By sedimentation velocity, we observed a symmetric peak that indicated that the mixtures were homogeneous.

In the 280% supersaturated solutions, it was impossible to determine the apparent molecular weight because these solutions were turbid and heterogeneous. In contrast, for micelles supersaturated with cholesterol, the apparent molecular weight remained almost constant for cholesterol concentration ranges from 0.49 to 0.74 mM.

Physical state of supersaturated (280%) systems. Mixtures containing sodium taurocholate (10 mM), monoolein (10 mM) and oleate (20 mM) labeled with [^{14}C]oleic acid were opalescent. Examined under a light microscope, they appeared polydisperse. By centrifugation of 100,000 g for 90 min at 25 C, a separation into two phases occurred. The upper phase, turbid and rich in oleate and monoolein, represented about 10% of the total volume and, under polarized light microscopy, showed a texture consistent with the presence of liquid crystals with myelin figure formations. The lower phase, containing 80% of the total oleate, exhibited a Tyndall phenomenon and could pass through a membrane of 0.22 μm porosity. This phase is similar to a mixture supersaturated with lipids (230%) when compared to the micellar solubility limits. We have also generated mixtures containing oleate (14 mM) and monoolein (7 mM) in phosphate buffer, pH 6.7, 0.15 M Na^{+}. The emulsion contained droplets, but no paracrystalline phase could be detected. Upon centrifugation, an upper oil phase could be easily separated from a lower lipid-free aqueous phase. In comparing these two solutions, we concluded that the presence of bile salt allowed the dispersion of an amount of lipid larger than that incorporated in the mixed micelles.

Cholesterol uptake. The cholesterol uptake from everted jejunal sacs of rat intestine was studied from saturated (0.49 mM cholesterol) and supersaturated (0.64 mM and 0.78 mM cholesterol) solutions with 10 mM taurocholate and 8 mM oleate/monoolein.

The results for an incubation time of 5 min are given in Table 2. We observed an increase of cholesterol uptake directly proportional to the quantity of cholesterol solubilized in the mixtures. The difference of uptake is significant ($2p \leqslant 0.05$) between the saturated solutions and the 130% supersaturated solutions and between the 130% and 150% supersaturated solutions.

Oleate uptake. The oleate uptake was measured from saturated (10.55 mM oleate/monoolein) and from supersaturated solutions (13.70, 15.80 and 30.00 mM oleate/monoolein) with 10 mM taurocholate.

As for cholesterol uptake, the oleate uptake was determined after a 5-min incubation time. Control studies showed a constant uptake rate between 3- and 15-min incubation times. The results, presented in Table 3, showed that the oleate uptake is almost constant from saturated and supersaturated solutions, regardless of the quantity of oleate solubilized in the mixtures. This value corresponds therefore to the maximum uptake in our conditions. As shown in Table 3, we verified that the uptake from less saturated solutions (60%) is smaller.

TABLE 2

Cholesterol Uptake after Cholesterol Supersaturation of Mixed Micelle Taurocholate/Oleate/Monoolein/Cholesterol

Cholesterol concentration (mM)	Saturation (%)	n	Cholesterol uptake (nmol·100 mg^{-1}·5 min^{-1})	Adherent fluid volume (μl·100 mg^{-1}·5 min^{-1})
0.49	100	36	77.39 ± 2.17	72.63 ± 3.49
0.64	130	15	92.59 ± 6.30*	77.98 ± 4.99
0.74	150	26	120.59 ± 4.04*	62.03 ± 3.37

The taurocholate concentration (10 mM) and oleate/monoolein concentration (8 mM) are kept constant.
*Indicates a significant difference ($P < 0.05$) between the mean values of cholesterol uptake from saturated and supersaturated solutions.
Each value represents the mean ± SE; n is the number of samples.

TABLE 3

Oleate Uptake after Oleate Supersaturation of Mixed Micelle Taurocholate/Oleate/Monoolein

Oleate/monoolein (mM)	Saturation (%)	n	Oleate uptake (nmol·100 mg^{-1}·5 min^{-1})	Adherent fluid volume (μl·100 mg^{-1}·5 min^{-1})
6.33	60	12	837 ± 36*	60.11 ± 7.31
10.55	100	45	1256 ± 21	76.74 ± 2.96
13.70	130	32	1334 ± 41	87.49 ± 3.08
15.80	150	18	1340 ± 49	75.18 ± 4.51
30.00	280	39	1158 ± 50	62.11 ± 2.82

The taurocholate concentration is kept constant at 10 mM.
*Indicates a significant difference ($p < 0.005$) between the mean values of oleate uptake from unsaturated and saturated or supersaturated solutions.
Each value represents the mean ± SE; n is the number of samples.

TABLE 4

Intestinal Oleate Uptake from Saturated Taurocholate/Oleate Micelle

Micelle	n	Oleate uptake (nmol·100 mg^{-1}·5 min^{-1})	Adherent fluid volume (μl·100 mg^{-1}·5 min^{-1})
A[a]	12	1724 ± 78	84.42 ± 4.55
B[b]	12	2020 ± 52*	77.63 ± 3.50

[a]Micelle taurocholate/oleate (10 mM/9 mM).
[b]Micelle taurocholate/oleate (15 mM/13.5 mM).
*Indicates a significant difference ($P < 0.005$) between the mean values of oleate uptake from A and B micellar solutions.

The maximal uptake rate was not due to a saturation of the mucosal surface of the everted sacs. Oleate uptake was increased further when using saturated solution with 15 mM taurocholate or 10 mM taurocholate containing oleate without monoolein (Table 4).

DISCUSSION

In our studies, the intestinal uptake of lipids was measured in vitro using everted intestinal sacs, and it was necessary to account for possible drawbacks of this model. First, under our experimental conditions, there was no apparent damage of the mucosa when we used taurocholate more than 98% pure. This bile salt has been shown to be without any toxicity for the intestinal mucosa at 10 mM (8), which is lower than the bile salt concentration used in other studies of intestinal uptake (9). This lack of toxicity, even in the presence of high concentrations of fatty acids and monoolein, is further demonstrated by the fact that the mucosal permeability for macromolecules seems to be essentially unchanged by any of the test solutions used in the present work. The adherent fluid volume estimated from [^{3}H]inulin would be increased in case of increased cellular permeability. In

all of our experiments, it was the same as the volume measured with control solutions without bile salts or fatty acids.

It must be emphasized that the adherent fluid volume calculated from inulin binding was not dependent on the amount of lipid present in the bulk phase. This suggests that even the use of 280% supersaturated solutions does not preclude the use of inulin as a marker of extracellular fluid. Another possible limitation of the study was the short time (5 min) during which uptake rates were measured. This could represent only the initial rate of uptake, possibly different from the rate at equilibrium, particularly when supersaturated solutions with a slower diffusion through the unstirred water layer are studied. This did not seem to be the case, as fatty acid uptake remained linear for at least 15 min in even the most supersaturated solutions.

On the other hand, additional experiments showed that oleate uptake was not limited by the ability of intestinal mucosa to bind oleate. Indeed, oleate uptake may be increased above the values obtained in these experiments when more oleate is presented in micellar form (taurocholate 15 mM or oleate/bile salt micelles without monoolein). In fact, the major limitation of the study is that we cannot know if any of the solutions are actually similar to the lipidic phases occurring during fat absorption in vivo. Many other molecules, and particularly proteins and calcium, are present during fat absorption. For solutions supersaturated with oleate and monoolein, however, microscopic examination showed structures that seem analogous to the ones seen during fat digestion (1).

Ultracentrifugation of these mixtures did not show a distinct separation between the clear aqueous phase and the upper lipidic phase rich in liquid crystalline materials. In the clear aqueous phase, the presence of a viscous isotropic phase was shown by microscopic examination exactly as in intestinal contents (1). Under these conditions, these phases seem to have physiological implications, even if the conclusions of the study are obviously valid only for our experimental conditions.

We also observed different results for cholesterol and fatty acid uptake. For cholesterol, the uptake rate increases with cholesterol concentration (10), even for cholesterol supersaturated solutions. Furthermore, the same results have recently been obtained elsewhere (8). This suggests that supersaturation with cholesterol does not induce the formation of oleate/monoolein/cholesterol microdroplets similar to the structures described in dilute bile salt/lecithin/cholesterol supersaturated solutions (11). More likely, the supersaturation state results in a metastable micelle from which cholesterol absorption occurs. This would be consistent with our results, because the size of mixed micelles is not dependent on their cholesterol content, and it has been shown that cholesterol uptake from mixed micelles of a given micellar size increases linearly with their cholesterol content (12).

The results are very different for fatty acids, whose uptake rate from saturated mixed micelles cannot be increased by supersaturation. This is consistent with a role of both micellar and nonmicellar structures in fatty acid absorption for the following reasons: (i) Slightly supersaturated solutions (130% or 150% saturation) are either optically clear or slightly Tyndall+. During analytical ultracentrifugation, they appear to contain homogeneous-sized particles larger than saturated micelles, but oleate uptake from these solutions is similar to oleate uptake from saturated micelles. This shows that diffusion across the unstirred water layer is possible for structures larger (two- or threefold) than saturated mixed micelles. It may be hypothesized that the high concentration of fatty acids compensated for a slower aqueous diffusion of aggregates (10) so that the net result was the same uptake as in the presence of nonsupersaturated micelles. (ii) The role of nonmicellar phases is more clearly shown by the fact that turbid solutions with bile salts and a high concentration of fatty acids and monoolein deliver fatty acids at the same rate as micelles. These solutions are polydisperse solutions containing liquid crystalline phases and saturated micelles with structures of intermediary size as shown by microscopic examination after ultracentrifugation.

The same polydispersity has been observed for bile salt/lecithin mixtures (11) where the bile salts are involved in the formation of paracrystalline phases. The microscopic appearance of the largely supersaturated bile salt/oleate/monoolein mixtures cannot be explained by a simple coexistence of saturated mixed micelles and an emulsion of oleate/monoolein in water. This is clearly shown by the study of sonicated emulsions of oleate/monoolein at concentrations equal to the difference between total lipid concentration of the supersaturated solutions and the concentration corresponding to saturation of the micelles. In such solutions, the lipidic phase separates clearly from the aqueous phase and forms neither liquid crystalline nor viscous isotropic phases. It seems then that in our solutions bile salts are partitioned between mixed micelles and other phases. Under these conditions, intestinal uptake from micelles is certainly lower than that from pure micellar solutions containing the same amount of bile salts as the total concentration present in the mixtures. Since the net uptake is, in fact, the same as from pure saturated mixed micelles, the results suggest that part of the intestinal uptake is due to absorption from other liquid crystalline phases than micelles.

It is obviously difficult to know if a similar phenomenon occurs during digestion in vivo. Some of the solutions studied show the same microscopic structures as seen during in vivo lipolysis. They have approximately the same lipid composition as that measured in the duodenal aqueous phase during digestion of lipids (13–15). However, it must be emphasized that many other components not studied here, such as calcium and proteins, may interfere with lipid absorption in vivo.

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Intestinal Absorption of Ester and Ether Glycerophospholipids in Guinea Pig. Role of a Phospholipase A_2 from Brush Border Membrane

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In vivo intestinal perfusion was used to follow the absorption of three different choline glycerophospholipids (CGP) in guinea pig. These included 1-[^{3}H]palmitoyl-2-acyl-*sn*-glycero-3-phosphocholine (diacyl-GPC), 1-[^{3}H]-*O*-hexadecyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) and 1,2-di-*O*-hexadecyl-*sn*-glycero-3-phospho-[^{3}H]-choline (dialkyl-GPC). About 80% of diacyl-GPC was absorbed within 4 hr, compared to 60% of alkylacyl-GPC and 30% of dialkyl-GPC. The radioactivity disappearing from the perfusion fluid was recovered in intestinal lipids, mostly triacylglycerol, free fatty acid and CGP from diacyl-GPC, CGP from alkylacyl-GPC and dialkyl-GPC. These results indicated that the nonhydrolyzable substrate dialkyl-GPC was much less absorbed, whereas diacyl-GPC, which released over 80% of [^{3}H]palmitic acid in the perfusion fluid, displayed the highest absorption rate. The intermediate picture observed for alkylacyl-GPC suggested the possible involvement of a phospholipase A_2, which was detected in the entire intestinal tract. This enzyme was further found to concentrate in villus cells, where it is localized in the brush border membrane, as shown using two different subcellular fractionation procedures. These data suggest a possible role of this new enzyme in the digestion of alimentary phospholipids.
Lipids 22, 33–40 (1987).

It is generally accepted that intestinal absorption of glycerophospholipids requires hydrolysis in the intestinal lumen (1,2). Such lipolysis is achieved by pancreatic phospholipase A_2, which specifically removes the fatty acids esterified in the *sn*-2-position of phosphoglycerides (3,4). Further degradation of the lysophospholipids involves pancreatic lysophospholipases (5), but this remains limited to 1-acyl-compounds, since the 1-*O*-alkyl- and the 1-*O*-alkenyl bonds of ether phospholipids resist hydrolysis. Ether glycerophospholipids can represent a substantial portion of phospholipids present in food (6–8). Following hydrolysis, free fatty acids (FFA) and lysophospholipids are absorbed as such through the brush border membrane (1). Guinea pig might well represent an exception to this general scheme, because the secretory pancreatic phospholipase A_2 is absolutely lacking in this species (9), in contrast to the presence of two cationic lipases with high phospholipase A_1 activities (10–12).

The present study was undertaken to investigate comparatively the intestinal absorption of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC), 1,2-diacyl-*sn*-glycero-3-phosphocholine (diacyl-GPC) and 1,2-di-*O*-alkyl-*sn*-glycero-3-phosphocholine (dialkyl-GPC) using in vivo perfusion of the guinea pig small intestine. The relationship between phospholipid degradation and absorption led us to describe a phospholipase A_2 in guinea pig intestinal brush border membrane, which could play a significant role in the digestion of alimentary phospholipids.

MATERIALS

Guinea pigs (300–400 g) were obtained from a local distributor. [9-10(n)^{3}H]Palmitic acid (500 mCi/mmol), 1-[^{14}C]linoleic acid (60 mCi/mmol) and [^{14}C]methyl iodide (58 mCi/mmol) were purchased from the Radiochemical Center (Amersham, United Kingdom) and L-α-kephalin (N,N-dimethyl dihexadecyl) was from Serva (Heidelberg, Federal Republic of Germany). Vitride [$NaAlH_2(OCH_2CH_2OCH_3)_2$] was obtained from Labolac (Rueil-Malmaison, France). All other chemicals were from Prolabo (Paris, France) or Merck (Darmstadt, Federal Republic of Germany). The composition of Ringer Locke solution used for intestinal perfusion was as follows: 116 mM NaCl, 20 mM $NaHCO_3$, 5.2 mM KCl, 2.4 mM Na_2HPO_4, 0.4 mM NaH_2PO_4, 1.2 mM $MgCl_2$, with pH adjusted to 7.4 by bubbling a 95% O_2/5% CO_2 mixture. Final osmolarity was between 285 and 318 mOs.

METHODS

Radioactive phospholipids. 1-[^{3}H]Palmitoyl-2-acyl-GPC (13,900 dpm/nmol) and 1-acyl-2-[^{14}C]linoleoyl-GPC (1,500 dpm/nmol) were prepared using rat liver microsomes as described by Waite and van Deenen (13) and van den Bosch et al. (14). 1-[^{3}H]Hexadecyl-2-acyl-GPC (2,040 dpm/nmol) was obtained by the procedure described by Wykle et al. (15,16) with the following modifications: [^{3}H]hexadecanol prepared by Vitride reduction of [^{3}H]-palmitic acid (30 min) was injected intraperitoneally into mice bearing Krebs II ascitic tumor. After 24 hr, ascites cells were collected and their lipids extracted (17). Total choline glycerophospholipids (CGP) purified by thin layer chromatography (TLC) (18) were treated for 7 hr at 41 C with partially purified guinea pig phospholipase A_1 as described previously (8,19). 1-*O*-[^{3}H]Alkyl-2-acyl-GPC was purified by preparative TLC in the basic solvent of Broekhuyse (20). Before the migration, plates were exposed to HCl fumes to hydrolyze plasmalogen (19). 1,2-*O*-Dihexadecyl-*sn*-glycero-3-phospho-[^{14}C-*N*-methyl]choline (115,000 dpm/nmol) was synthesized by the methylation of the corresponding *N,N*-dimethyl-ethanolamine glycerophospholipid with [^{14}C]methyl iodide as described by Stoffel (21). It was then purified by preparative TLC on Silica Gel G plates, 0.25 mm thick (Merck) using chloroform/methanol/water (65:24:4, v/v/v) as a solvent.

Animal experimentation. Intestinal absorption of phospholipids was performed by closed circuit in vivo perfusion (22,23). Nonfasted guinea pigs were anaesthetized with sodium pentobarbital (0.5 ml/kg body weight). After tracheotomy and laparotomy of each animal, the small intestine was ligatured both at the level of the pylorus and after the first intestinal loop. Then two cannula made with polyvinyl tubing were inserted at both ends, and the isolated intestine part was rinsed twice with Ringer Locke solution at 37 C and connected to the perfusion system. For maximal lipid absorption, the flow rate was main-

tained at 1.7 ml/min. During the perfusion, animal body temperature was kept at 37 C with a thermostated plate. CGP (500,000 dpm/245 nmol) were taken to dryness under nitrogen from a chloroform/methanol solution and dispersed by sonication in 10 ml of Ringer Locke solution containing 6 mM sodium taurocholate. The percentage of substrate absorbed was evaluated by withdrawing 0.1 ml of perfusion solution after 15 min and then every 30 min up to 2 or 4 hr. These aliquots were directly put into vials containing 10 ml Aqualuma (Kontron, France), and their radioactivity was determined.

Extraction and analysis of lipids. At the times indicated in Tables 1 and 2, the animals were killed by decapitation. The part of perfused intestine was removed, and the perfused solution was collected. The intestine was washed thoroughly with Ringer solution containing 10 mM EDTA, and its lipids were extracted and washed according to Folch et al. (24). The perfused solution was diluted with EDTA (*10 mM final concentration*), and lipids were extracted according to Bligh and Dyer (17). Lipids were separated by TLC on Silica Gel G plates using hexane/diethyl ether/formic acid (55:45:1, v/v/v) for neutral

TABLE 1

Recovery of Radioactivity from Perfused Solution in Phospholipids and Neutral Glycerides after Administration of Different Labeled Substrates as Indicated in Materials and Methods[a]

	1-[^{3}H]Palmitoyl-2-acyl-GPC			1-[^{3}H]Hexadecyl-2-acyl-GPC			1,2-Dihexadecyl-[^{14}C]methyl-GPC	
Lipids[b]	0 hr	2 hr	4 hr	0 hr	2 hr	4 hr	0 hr	4 hr
CLGP	1.2 ± 0.5	3.8 ± 1.7	9.0 ± 0.4	9.7 ± 0.1	68.5 ± 6.5	63.4 ± 1.1	0.6 ± 0.6	0
SPH	0	0.2 ± 0.3	0.2 ± 0.2	0	0.8 ± 0.1	0.6 ± 0.5	0	
CGP	98.6 ± 0.6	4.0 ± 1.6	7.5 ± 0.1	89.7 ± 0.1	12.8 ± 3.6	17.2 ± 0.9	99.1 ± 0.5	98.4 ± 1.9
SGP + IGP	0	2.1 ± 1.8	1.9 ± 0.4	0	2.1 ± 1.2	2.9 ± 1.8	0	0
EGP	0	1.0 ± 0.4	3.1 ± 0.3	0	3.5 ± 3.8	1.4 ± 0.8	0	0
FFA	0.2 ± 0.2	54.2 ± 4.6	55.3 ± 1.4	0.6 ± 0.1	2.7 ± 1.5	2.1 ± 1.2	0.4 ± 0.3	0.1 ± 0.1
MG	0	11.3 ± 1.8	7.6 ± 0.2	0	2.7 ± 1.4	5.6 ± 2.0	0	0.8 ± 1.0
DG	0	6.7 ± 2.0	9.7 ± 2.0	0	1.2 ± 0.5	3.3 ± 0.7	0	0.5 ± 0.5
TG	0	12.3 ± 3.5	4.2 ± 0.8	0	5.0 ± 1.0	3.2 ± 1.3	0	0.3 ± 0.5
CE	0	3.7 ± 1.9	1.6 ± 1.2	0	0.6 ± 1.0	0.2 ± 0.3	0	0
Total	100	99.4	100.1	100	99.9	99.9	100.1	100.1

[a]Data presented are mean ± SEM of four values (two experiments).

[b]CLGP, choline lysoglycerophospholipids; SPH, sphingomyelin; CGP, choline glycerophospholipids; SGP, serine glycerophospholipids; IGP, inositol glycerophospholipids; EGP, ethanolamine glycerophospholipids; FFA, free fatty acid; MG, monoacylglycerol; DG, diacylglycerol; TG, triacylglycerol; CE, cholesteryl ester.

TABLE 2

Recovery of Radioactivity from Perfused Intestine in Phospholipids and Neutral Glycerides after Administration of Different Labeled Substrates as Described in Materials and Methods[a]

	1-[^{3}H]Palmitoyl-2-acyl-GPC		1-[^{3}H]Hexadecyl-2-acyl-GPC		1,2-Dihexadecyl-[^{14}C]methyl-GPC
Lipids[b]	2 hr	4 hr	2 hr	4 hr	4 hr
CLGP	3.3 ± 2.5	3.0 ± 1.8	4.0 ± 2.1	1.0 ± 0.3	2.3 ± 1.0
SPH	1.8 ± 1.8	2.7 ± 1.6	0.6 ± 1.2	1.1 ± 0.3	0
CGP	17.4 ± 2.1	21.3 ± 1.6	74.1 ± 2.6	55.0 ± 5.8	88.4 ± 2.4
SGP + IGP	3.4 ± 1.9	3.8 ± 1.9	1.3 ± 1.7	3.6 ± 2.1	2.6 ± 2.6
ELGP	0.7 ± 0.8	0.9 ± 1.1	0.1 ± 0.2	2.0 ± 2.1	0
EGP	4.3 ± 1.0	7.7 ± 1.9	9.8 ± 1.0	23.7 ± 4.0	0.7 ± 0.8
FFA	22.0 ± 0.6	10.7 ± 2.4	1.1 ± 0.5	1.5 ± 0.4	0.8 ± 0.3
MG	3.7 ± 1.2	2.4 ± 0.3	1.6 ± 1.0	1.9 ± 0.4	3.0 ± 1.6
DG	9.3 ± 2.1	8.8 ± 1.7	4.4 ± 2.8	6.2 ± 2.5	0.5 ± 0.7
TG	30.2 ± 1.5	33.1 ± 2.2	1.5 ± 0.5	3.3 ± 0.3	1.1 ± 1.2
CE	0.6 ± 0.5	0.8 ± 0.7	0.9 ± 0.4	0.7 ± 0.4	0.4 ± 0.4
X[c]	2.7 ± 1.0	4.5 ± 1.5	0.5 ± 0.7	0.4 ± 0.2	0.3 ± 0.5
Total	99.4	99.7	99.9	100.4	100.1

[a]Data presented are mean ± SEM of four values (two experiments).

[b]ELGP, ethanolamine lysoglycerophospholipids; see Table 1 footnote for other abbreviations.

[c]X is a substance migrating between FFA and TG.

glycerides (25) and chloroform/methanol/acetic acid/water (65:43:1:3, v/v/v/v) for glycerophospholipids (18). After visualization with iodine vapor, the different lipid spots were scraped off and their radioactivity was determined.

Preparation of mucosal homogenates and subcellular fractionation. Animals fasted for 12 hr were killed by a blow to the head and the small intestine was entirely removed. It was rinsed with ice-cold isotonic NaCl, and the mucosa was homogenized on ice in 20 vol Tris-Sucrose buffer (250 mM sucrose buffered with 10 mM Tris-HCl, pH 7.3) using a Potter Elvejhem homogenizer (26). All the subsequent operations were performed on ice or at 4 C. The homogenate was then subjected to a sequential centrifugation procedure, i.e., 900 $\times$ g for 10 min, 10,000 $\times$ g for 10 min and 100,000 $\times$ g for 90 min. At each step the resulting supernatant was carefully removed and used for the following centrifugation, except the last one (cytosolic fraction). The three pellets were resuspended in Tris-Sucrose buffer using a Potter Elvejhem homogenizer.

In some experiments, the whole small intestine was divided into three equal segments, corresponding to the duodenojejunal area, to the remaining jejunum and to the ileum, from the proximal to the distal part.

Purification of brush border membrane vesicles. This was achieved exactly as described by Schmitz et al. (27).

Isolation of various intestinal cell populations. Intestinal cells were isolated and separated as a villus to crypt gradient according to Weiser (28). Cells were washed, suspended in 300 mM mannitol buffered with 10 mM Tris-HCl (pH 7.3) and used for assay of alkaline phosphatase and phospholipase A_2 activities.

Determination of phospholipase activities. Three different substrates, prepared as described above, were used: 1-[^{3}H]palmitoyl-2-acyl-GPC, 1-acyl-2-[^{14}C]linoleoyl-GPC and 1-[^{3}H]hexadecyl-2-acyl-GPC. Depending on the case, each of them was taken to dryness under nitrogen from chloroform/methanol solution and dispersed by sonication (MSE sonicator at maximal output for 15 sec) in the assay mixture, which contained, in a final volume of 0.1 ml, 0.4 mM substrate, 4.8 mM sodium deoxycholate, 5 mM $CaCl_2$, 200 mM Tris-HCl (pH 7 and 8.5 for phospholipases A_1 and A_2, respectively, unless otherwise stated) and protein fractions from intestine. Incubation was carried out at 37 C for 15 min under shaking. The reaction was terminated by addition of chloroform/methanol and, after extraction (17), the lipids were separated by TLC on Silica Gel G plates using chloroform/methanol/water (65:35:4, v/v/v) as a solvent. After visualization with iodine vapor, the various spots corresponding to choline lysoglycerophospholipids (CLGP), CGP and FFA were scraped off and radioactivity was determined.

Other marker enzymes. The activity of the following marker enzymes was determined according to well-established procedures: alkaline phosphatase (29), succinate cytochrome c reductase (30), N-acetyl-β-D-glucosaminidase (31), NADH-dehydrogenase (32,33), lactate dehydrogenase (34) and sucrase (35).

Other analytical methods. Protein was determined by the method of Lowry et al. (36) using bovine serum albumin as a standard. Lipid phosphorus was measured using a modification of the Fiske and Subbarow method (37). Radioactivity was evaluated with a Kontron Intertechnique Liquid Spectrometer (type SL 400) with automatic quenching correction, using Aqualuma as a scintillation fluid.

RESULTS

Intestinal absorption and metabolism of various radioactive CGP. The rate of radioactivity disappearance from the perfusion solution indicated intestinal absorption of phospholipid, since no decrease in radioactivity present in the perfusion fluid was observed during perfusion in the same system lacking the intestinal segment. Figure 1 shows that both 1-[^{3}H]palmitoyl-2-acyl-GPC and 1-[^{3}H]-hexadecyl-2-acyl-GPC were absorbed through guinea pig intestinal mucosa, although 40% of [^{3}H]alkylacyl-GPC was still present in the perfused solution after 4 hr, compared to 20% of diacyl-GPC. Dialkyl-GPC disappeared to a much lower extent under the same conditions, since 65–70% of the initial radioactivity was recovered in the perfusion fluid after 4 hr.

To study the differences in phospholipase susceptibility of the three substrates, the distribution of radioactivity between the various lipid classes was investigated in the perfused solution. As shown in Table 1, both 1-[^{3}H]palmitoyl-2-acyl-GPC and 1-*O*-[^{3}H]hexadecyl-2-acyl-GPC were almost entirely hydrolyzed into [^{3}H]palmitic acid and 1-*O*-[^{3}H]hexadecyl-2-lyso-GPC, respectively. However, some 12–18% of the alkylacyl compound remained unchanged, whereas no evident degradation could be detected for 1,2-dihexadecyl-[^{14}C]choline-GPC, which resisted hydrolysis by guinea pig phospholipase A_1 and pig pancreas phospholipase A_2 (this was verified during in vitro incubations).

Upon extraction of lipids from the whole intestinal wall, about 12 $\pm$ 1%, 31.6 $\pm$ 5.6% and 4.5 $\pm$ 1.5% of the initial radioactivity were recovered from 1-[^{3}H]palmitoyl-2-acyl-GPC, 1-*O*-[^{3}H]alkyl-2-acyl-GPC and 1,2-dihexadecyl-[^{14}C]choline-GPC, respectively (mean $\pm$ SEM, three determinations). As shown in Table 2, most of the radioactive

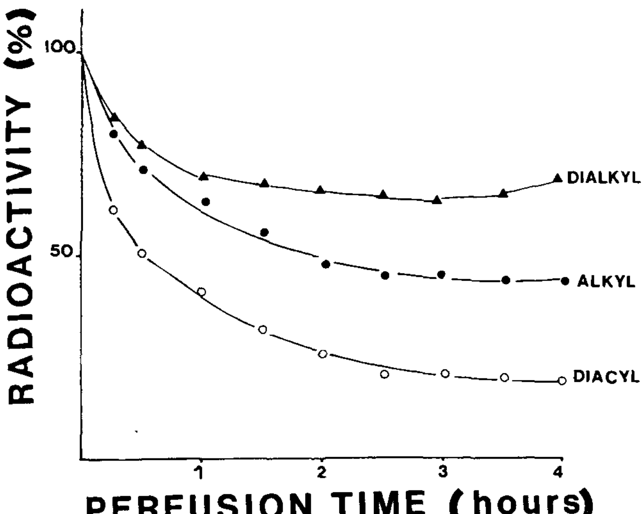


FIG. 1. Rate of disappearance of radioactivity from the perfused solution during intestinal perfusion of 1-[^{3}H]palmitoyl-2-acyl-GPC (○) 1-*O*-[^{3}H]hexadecyl-2-acyl-GPC (●) and 1,2 di-*O*-hexadecyl[^{14}C]-methyl-GPC (▲). The values are means of two experiments for each choline glycerophospholipid.

palmitic acid was detected in TG, CGP and FFA, which represented 33%, 21% and 11% of the total intestinal radioactivity, respectively. Moreover, comparison of data obtained at 2 and 4 hr indicated that some free palmitic acid was progressively incorporated into CGP, ethanolamine glycerophospholipids (EGP) and TG. In contrast, the radioactivity derived from 1-*O*-[^{3}H]hexadecyl-2-acyl-GPC was mostly detected in CGP, although some of it was converted into EGP and TG between 2 and 4 hr perfusion.

Finally, dihexadecyl-[^{14}C]choline-GPC displayed a higher metabolic stability, since 83% of the radioactivity was still present with CGP after 4 hr. It was also hardly converted into diacylglycerol (DG), which still represented 8-10% of the total radioactivity associated with the two other substrates.

Studies on intestinal phospholipases A. The observation that alkylacyl-GPC was better absorbed than dialkyl-GPC, owing to hydrolysis of the former during intestinal perfusion, prompted us to investigate the presence of a phospholipase A_2 in guinea pig intestine. Indeed, incubation of intestinal homogenates at pH 7.0 with 1-acyl-2[^{14}C]linoleoyl-GPC led to the release of [^{14}C]linoleic acid. However, this was accompanied by the simultaneous formation of 2-[^{14}C]linoleoyl-GPC, indicating the concomitant action of a phospholipase A_1. These results were confirmed by data showing that both [^{3}H]palmitic acid and 1-[^{3}H]palmitoyl-GPC were generated under the same conditions when using 1-[^{3}H]palmitoyl-2-acyl-GPC as a substrate. As shown in Figure 2, the two phospholipase activities were stimulated to the same degree by sodium deoxycholate, with optimal activation being attained at 5 mM of the detergent. Moreover, omission or addition of calcium (5 mM) did not modify significantly the results and did not allow discrimination between the two phospholipases A (not shown).

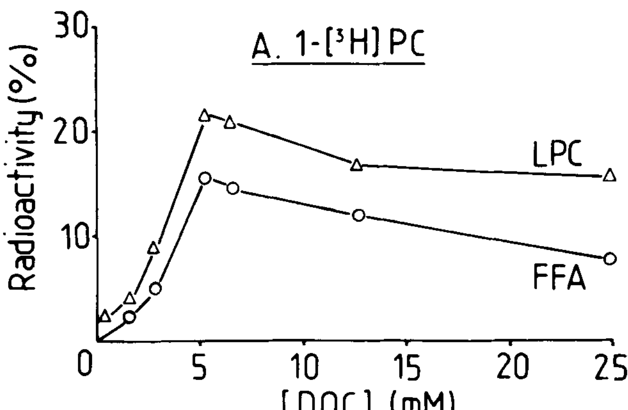
However, the two enzyme activities could be partly separated by submitting intestinal homogenate to subcellular fractionation. As illustrated in Figure 3, four different fractions could be isolated and characterized, based on their content of various marker enzymes: alkaline phosphatase (brush border membrane; 27) in the 900 × g fraction; succinate cytochrome c reductase (mitochondria; 38) and N-acetyl-β-D-glucosaminidase (lysosomes; 31,38) in the 10,000 × g fraction; NADH-diaphorase (endoplasmic reticulum; 32,33,38) in the microsomal fraction; and lactate dehydrogenase (cytosol; 34,38) in the final supernatant. Figure 3 also indicates that phospholipase A_2 activity displayed the same distribution as alkaline phosphatase, especially considering the generation of 1-acyl- or 1-alkyl-GPC, whereas phospholipase A_1 activity displayed the behavior of a cytosolic enzyme.

Such a dual distribution of the two enzymes became more evident upon comparing their pH dependence in the cytosolic fraction and the 900-×-g sediment. As shown in Figure 4A, phospholipase A_1 activity (as detected from the release of [^{3}H]palmitic acid or 2-[^{14}C]linoleoyl-GPC from the corresponding substrate) predominated in the cytosol and displayed a broad optimal pH range between 6.5 and 10, culminating at 7.5. In contrast, the major enzyme activity in the 900-×-g fraction was phospholipase A_2 (as deduced from the liberation of [^{14}C]linoleic acid and 1-[^{3}H]palmitoyl-GPC from their respective precursors), which displayed a stronger pH dependence with a peak at 8.5 (Fig. 4B).

As indicated in Figure 5, other differences between the two phospholipases A included a relatively higher thermostability of phospholipase A_2 vs phospholipase A_1, with the latter displaying the behavior previously described for the pancreatic enzyme (9).

These results suggested a localization of guinea pig intestine phospholipase A_2 in the brush border membrane. Moreover, as shown in Figure 6, sequential elution of intestinal cells according to the procedure described by Weiser (28) revealed that phospholipase A_2 activity decreased from villi to crypts in a manner similar to alkaline phosphatase. Finally, phospholipase A_2 activity was determined in microvesicles purified from brush border membranes (Table 3). Although some differences appeared depending on the substrate used, in all cases phospholipase A_2 activity was enriched in microvesicles to the same extent as sucrase, a very specific marker of brush border membrane (27,35).

To gain further insight into the possible physiological role of this phospholipase A_2, its activity was measured in different segments isolated from guinea pig small intestine. Data presented in Table 4 indicates that the enzyme was distributed throughout the small intestinal tract.



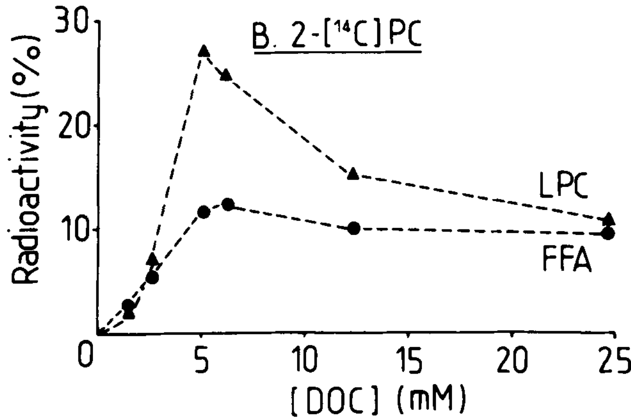


FIG. 2. Effect of sodium deoxycholate on phospholipase activities from homogenates of guinea pig small intestine. Data are expressed in percentages of radioactivity recovered in lysophosphatidylcholine (LPC) or free fatty acid (FFA) using 1-[^{3}H]palmitoyl-2-acyl-GPC (A) or 1-acyl-2-[^{14}C]linoleoyl-GPC (B) as substrates. DOC, sodium deoxycholate.

DISCUSSION

Our results confirm the general view that there is a direct relationship between the absorption level of a given phospholipid and its susceptibility to hydrolysis. This is particularly clear in the case of 1,2-dihexadecyl-GPC, which should not be degraded in the intestinal lumen,

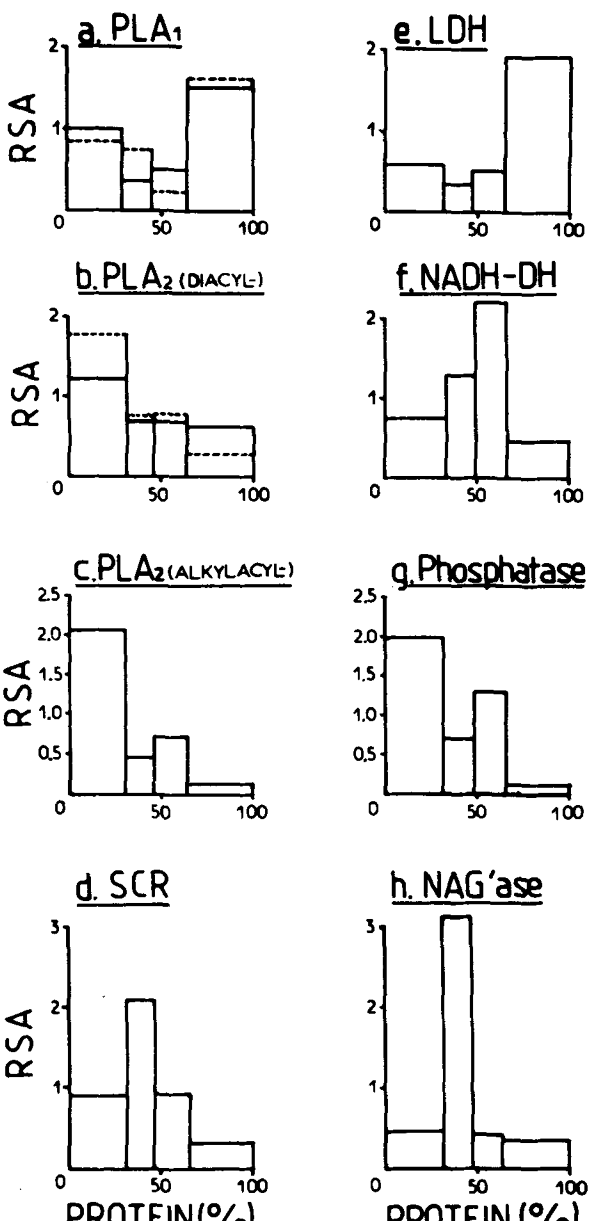


FIG. 3. Distribution of various marker enzymes and phospholipases A in different subcellular fractions isolated from guinea pig small intestine. Relative specific activities (RSA) were calculated according to de Duve et al. (38). (a) Phospholipase A_1, detected by the release of [^{3}H]palmitic acid from 1-[^{3}H]palmitoyl-2-acyl-*sn*-GPC (——) or by the generation of 2-[^{14}C]linoleoyl-GPC from 1-acyl-2-[^{14}C]linoleoyl-GPC (– – –); (b) phospholipase A_2 detected by the liberation of [^{14}C]linoleic acid (——) or by the formation of 1-[^{3}H]palmitoyl-GPC (– – –) from the corresponding substrates; (c) phospholipase A_2, detected by the release of 1-[^{3}H]hexadecyl-GPC from 1-[^{3}H]hexadecyl-2-acyl-GPC; (d) succinate cytochrome c reductase (SCR); (e) lactate dehydrogenase (LDH); (f) NADH dehydrogenase (NADH-DH); (g) alkaline phosphatase; (h) N-acetyl-β-D-glucosaminidase (NAG'ase).

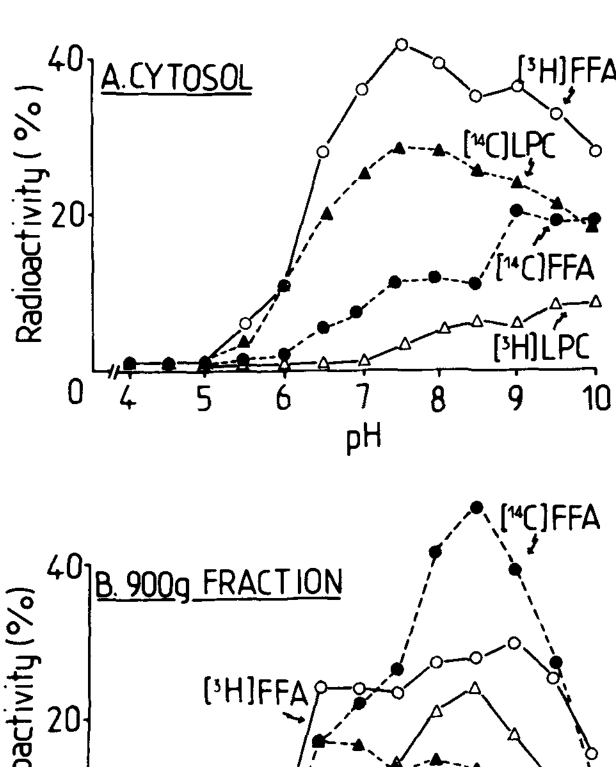


FIG. 4. The pH dependence of phospholipase A activities present in the cytosol (A) and the 900-g pellet (B) isolated from guinea pig small intestine. Buffers were 0.2 M citrate-phosphate for pH 4 to 6.5 and 0.2 M Tris-HCl for pH 7 to 10.

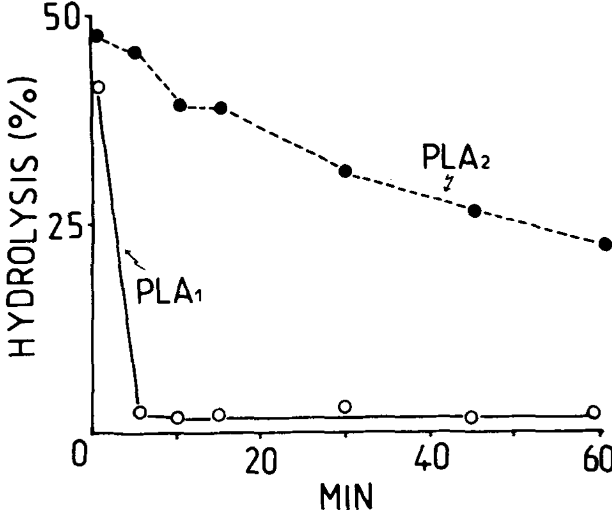


FIG. 5. Effect of heat treatment on the activities of phospholipase A_1 from intestine cytosol (PLA_1) and of phospholipase A_2 from the 900-X-g fraction (PLA_2). Enzyme fractions were treated at 60 C for the indicated times, followed by determination of enzymatic activity. Phospholipase A_1 and A_2 activities were determined by the release of [^{3}H]palmitic acid and [^{14}C]linoleic acid from 1-[^{3}H]palmitoyl-2-acyl-GPC and from 1-acyl-2-[^{14}C]linoleoyl-GPC, respectively.

TABLE 3

Specific Activities of Sucrase and Phospholipase A_2 in Guinea Pig Intestine Homogenate and in Purified Microvesicles from Brush Border Membrane[a]

Enzymes	Specific activities		Enrichment (×-fold)
	Homogenate	Microvesicles	
Sucrase (nmol/min/mg)	206	2480	12
Phospholipase A_2 (nmol/min/mg)			
[^{14}C]Linoleic acid	20.4	204	10
1-[^{3}H]Palmitoyl-GPC	14.0	130	9
1-*O*-[^{3}H]Hexadecyl-GPC	9.0	100	11

[a]Phospholipase A_2 activity was determined using 1-acyl-2-[^{14}C]linoleoyl-GPC, 1-[^{3}H]palmitoyl-2-acyl-GPC and 1-*O*-[^{3}H]hexadecyl-GPC as substrates.

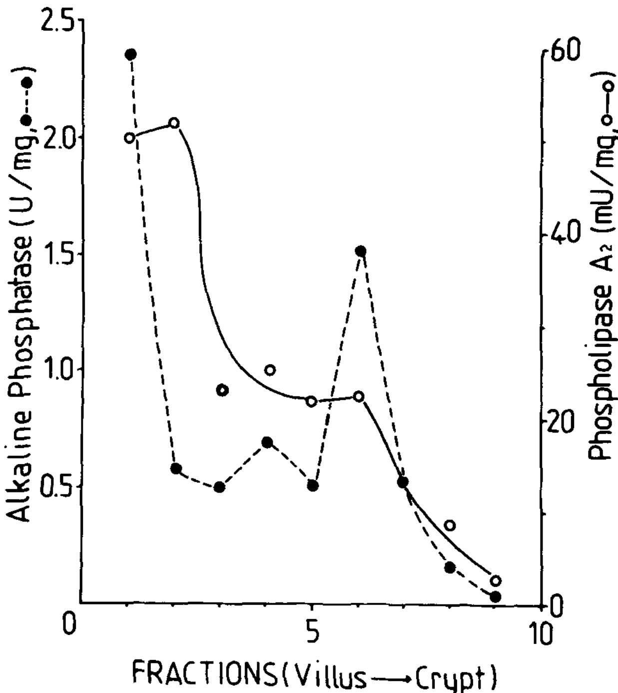


FIG. 6. Distribution of alkaline phosphatase and phospholipase A_2 along the crypt-villus complex. Phospholipase A_2 activity was determined using 1-acyl-2-[^{14}C]linoleoyl-GPC as a substrate. Results are from a typical experiment representative of three different experiments.

TABLE 4

Specific Activities of Phospholipase A_2 in Different Segments of Guinea Pig Small Intestine[a]

Segment[b]	Specific activities (nmol/min/mg)
Proximal	5.03 ± 0.53
Intermediary	9.60 ± 0.76
Distal	6.00 ± 1.96

[a]Phospholipase A_2 activities were determined using 1-acyl-2-[^{14}C]-linoleoyl-GPC as a substrate and are means ± SEM of three determinations.

[b]Proximal, intermediary and distal segments correspond to duodenojejunal area, remaining jejunum and ileum, respectively.

since no evidence for the presence of phospholipases C or D has yet been provided. In this respect, these results confirm previous findings that dialkylphospholipids are not hydrolyzed in the intestinal lumen of rat (39). One could argue that the disappearance of radioactive 1,2-dihexadecyl-GPC might simply reflect a passive adsorption onto the intestinal mucosa or some entrapment within the mucus. However, the radioactive phospholipid could not be removed by extensive washing.

The comparison between diacyl-GPC and alkylacyl-GPC leads to conclusions similar to those obtained by Paltauf (40) in the rat. Indeed, the latter compound is less absorbed than its diacyl analog. This absorption could even be somewhat less than observed, since it was not corrected for the presence of 10% lysocompounds in the substrate used (see Table 1). The lower absorption of alkylacyl-GPC vs diacyl-GPC could be explained by a different fate of the degradation products (lysophospholipid or FFA, respectively, rather than by a difference in the degree of hydrolysis (alkylacyl-GPC was almost entirely degraded).

Furthermore, the nature of the hydrolysis product could direct the metabolic fate of each phospholipid. Thus, palmitic acid is largely used by intestinal cells to resynthesize TG. In contrast, 1-hexadecyl-2-lyso-GPC is essentially reacylated into the corresponding phospholipid. It is tempting to speculate that the relative ratio of ester and ether lipids present in food could influence the lipid composition of chylomicrons and very low density lipoproteins formed in intestine from exogenous lipid (41).

As to the metabolism of 1-*O*-[^{3}H]alkyl-2-acyl-GPC, we found some evidence for a significant conversion into EGP and TG, with some accumulation of radioactivity in DG. Such a metabolic behavior is more difficult to explain than in the case of 1-[^{3}H]palmitoyl-2-acyl-GPC, where [^{3}H]palmitic acid is the direct precursor. One cannot exclude that the *O*-alkyl-bond was not cleaved in intestinal cells by a monooxygenase similar to that described in liver (42), followed by oxidation of hexadecanol to palmitic acid. In this respect, Paltauf (39) observed that 3 hr after oral administration of 1-*O*-[^{3}H]octadecyl-2-acyl-GPC, 93% of intestinal phosphatidylcholine was in diacyl-GPC.

However, it also could be possible that 1-*O*-[^{3}H]hexadecyl-2-acyl-GPC are converted into DG through the reverse action of CDP choline:DG cholinephosphotransferase (43,44) or by a phospholipase C acting on choline phosphoglycerides, as those described in the cytosol from rat brain (45) or from dog myocardium (46). This would provide DG, which could serve as a precursor for EGP. To discriminate between these possibilities, it would be necessary to analyze the various subclasses of intestinal CGP. However, this was not the aim of the present work.

The major purpose of this study was to investigate the intestinal absorption of natural ether glycerophospholipids in the guinea pig, chosen as a model because of the absence of pancreatic phospholipase A_2. Unexpectedly, 1-*O*-alkyl-2-acyl-GPC was absorbed at a rather good level; this involved its hydrolysis by a phospholipase A_2-like enzyme. Although intestinal phospholipases have been described in several species (26,47–53), their physiological role has not been emphasized. Such studies have been hampered by the fact that the small intestine is heavily contaminated by the high phospholipase A_2 activity originating from pancreas. Thus, the lack of phospholipase A_2 in guinea pig pancreas (9) offers significant methodological advantages over other species in investigations dealing with intestinal phospholipase A_2. Indeed, despite the contamination by a high phospholipase A_1 originating from the pancreatic gland, the present study provides clear evidence that guinea pig intestine contains a phospholipase A_2. This conclusion is supported by the observation that intestinal homogenates as well as purified fractions are able to generate 1-[^{3}H]palmitoyl-GPC and 1-[^{3}H]hexadecyl-GPC from the corresponding diradyl-GPC. However, determination of lysocompounds yields enzyme activities lower than those measured by following liberation of [^{14}C]linoleic acid from 1-acyl-2-[^{14}C]-linoleoyl-GPC. This might be explained by the fact that 1-[^{3}H]palmitoyl-GPC can be further degraded by the contaminating phospholipase A_1, which was shown to display a high lysophospholipase activity (12). As to the still lower activity measured with alkylacyl-GPC as a substrate, this might reflect some preferential hydrolysis of diacyl-GPC.

This new intestinal phospholipase A_2 seems to differ from the enzyme described by Mansbach et al. (51) in various mammalian species and purified by Verger et al. (52–53) from porcine small intestine. The latter phospholipase A_2 has been localized in Paneth cells situated in crypts from the jejunum (53), whereas the guinea pig intestine phospholipase A_2 is unambiguously and specifically located in the brush border membrane of the small intestine. Also, the pig enzyme proved almost inactive against phosphatidylcholine and displayed a strong preference for phosphatidylglycerol (51–53), in contrast to the phospholipase A_2 described herein, which was detected using CGP as substrates. Finally, the activity found in the guinea pig intestine does not seem to require calcium. Indeed, although $CaCl_2$ was routinely added to our incubation medium, its omission was without effect on the phospholipase A_2 activity, and so was the addition of EGTA. However, these results must be taken with some caution, since they were obtained with a crude enzyme preparation. Further studies on the purified enzyme are required before the new enzyme can be classified as belonging to a group of some recently described calcium-independent phospholipases A_2 (46,54).

ACKNOWLEDGMENTS

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Effect of Lecithin on the Release of 5′-Nucleotidase from Liver Plasma Membrane of Rat by Bile Acids

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Bile acids solubilize proteins from liver plasma membrane both in vivo and in vitro. The ability to solubilize the proteins is dependent on the species of bile acid. In this paper, the effect of phospholipid on the solubilization of a membrane-bound enzyme by bile acids was investigated in vitro. Taurocholate (TC) and tauroursodeoxycholate (TUDC) solubilized the enzyme, 5′-nucleotidase, from the liver plasma membrane of the rat in a concentration-dependent manner, although there was a great difference in their effect; at 40 mM, TC solubilized 55.4% of the original 5′-nucleotidase activity of the membrane, but TUDC only 5.7%. While lecithin alone had no solubilizing effect, its addition to the bile acids provoked a 10-fold increase in the solubilizing effect of TUDC, but virtually no change for TC, essentially equalizing the solubilizing effect of the two. Both TC-rich and TUDC-rich bile were obtained from rats infused with the respective bile acids via the jugular vein after their endogenous bile acid pool had been depleted. The solubilization effect of these biles was quite similar to the bile acid-lecithin mixtures. These findings demonstrate that lecithin enhances the ability of the bile acids to solubilize the membrane protein and eliminates the difference in the two bile acid species in their solubilizing ability.
Lipids 22, 41–44 (1987).

A large number of proteins is present in the bile of many mammalian species. It has been suggested that these proteins derive from serum and cells. Mullock et al. (1) indicated that some proteins in rat bile were immunologically related to proteins present in serum. Coleman and coworkers (2,3) showed that there were several plasma membrane-bound enzyme activities in bile. They also suggested that the presence of plasma membrane-bound enzymes in bile may be the result of bile salt action on the plasma membrane in the bile canaliculus.

Hatoff and Hardison (4), using bile-fistula rats, found that alkaline phosphatase, a typical plasma membrane enzyme, was secreted into bile in a bile acid species-dependent manner that was in the order taurodeoxycholate > TC > TUDC. Other investigators (5–7) also reported similar observations.

It is well known that bile acid exists in bile as a form of mixed micelles together with phospholipid and cholesterol (8,9). However, there is little information about the effect of phospholipid on the solubilization of protein from the membrane by bile acid. Therefore, in this paper, the ability of single micelles of bile acid and mixed micelles of bile acid-lecithin to solubilize 5′-nucleotidase from the hepatic plasma membrane of the rat in vitro are compared. TUDC was used because it causes minimal damage to cells and TC because it is the most common bile acid in rat bile.

MATERIALS AND METHODS

Animals. Nonfasted, male Fischer-344 rats weighing 200–350 g were used in all experiments.

Preparation of liver plasma membrane. Rat liver plasma membrane was prepared by the method of Boyer and Reno (10) with a slight modification, using 1 mM $NaHCO_3$ buffer (pH 7.5) containing 0.5 mM $CaCl_2$. The membrane was suspended in 1 mM $NaHCO_3$ buffer (pH 7.5) and stored in liquid nitrogen until used.

Release of 5′-nucleotidase from liver plasma membrane. The liver plasma membrane suspension was centrifuged at 6,000 × g for 10 min. The supernatant fluid was carefully removed. The resultant membrane precipitate was dispersed by pipetting at 4 C in 1 ml Tris-acetate buffer (pH 7.4) containing various concentrations of bile acid. This membrane suspension was incubated at 37 C for 10 min in a shaking water bath. An aliquot fraction of the suspension was removed and assayed for 5′-nucleotidase ("total") activity. The incubation was terminated by centrifugation at 6,000 × g for 10 min at 4 C. The supernatant was carefully separated from the precipitate and filtered through a 0.22 μm Millex-GV filter (Millipore Co., Bedford, Massachusetts). Then the filtrate was assayed for soluble 5′-nucleotidase ("soluble") activity. Relative activity of solubilized 5′-nucleotidase was expressed as percent ratio of the soluble activity to the total activity. When bile was used instead of bile acid solution, the activity of 5′-nucleotidase in the bile was subtracted from the activity in each fraction.

Assay of 5′-nucleotidase activity. The activity of 5′-nucleotidase was determined by the method of Nakamura and Kameyama (11) with slight modifications. The reaction mixtures contained 4 mM 5′-AMP, 4 mM $MgCl_2$, 100 mM Tris-acetate (pH 7.4) and the protein sample in a final volume of 0.5 ml. The reaction was run for 5 min at 37 C and terminated by addition of 1 ml of 8% trichloroacetic acid. Then 2 ml of $CHCl_3$ was added, mixed by vortexing, and centrifuged at 3,000 rpm for 5 min. One ml of the upper aqueous solution was used to determine inorganic phosphate.

Collection of rat bile. Rats were anesthetized by pentobarbital. The bile duct and the jugular vein were cannulated with PE-10 tubing, which was tunneled subcutaneously to emerge from the back of the animal. After the incisions were sutured, the rat was placed in a Bollman-type restraining cage with ad libitum access to food and water. Bile was drained overnight (ca. 14 hr) to deplete the endogenous bile acid pool. One of the bile acids in a 3% albumin solution (pH 7.4, 300 mOs) then was infused into the jugular vein at 0.27 μmol/100 g body weight/min by a constant infusion pump while collecting bile. The bile was collected for 8 hr after the first 30-min infusion. Concentrations of total bile acid and phospholipid in the bile obtained from rats infused with TUDC were 40.0 mM and 5.51 mM, respectively. TUDC was the main bile acid in the bile and 85.2% of the total bile acid (TUDC bile.)

In the case of infusion of TC, TC was 93.1% of total bile acid in the bile (TC bile), and concentrations of total bile acid and phospholipid were 32.3 mM and 5.31 mM, respectively.

Chemical determinations. Total bile acid in bile was determined using 3α-hydroxysteroid dehydrogenase (12).

Analysis of each bile acid in bile was carried out by high performance liquid chromatography (HPLC) with a Bile-pack column (JASCO, Tokyo, Japan) and an immobilized 3α-hydroxysteroid dehydrogenase, Enzymepack (JASCO; 13). Total lipid phosphorus in the bile was determined by the method of Bartlett (14). Protein in the hepatic plasma membrane was determined by the method of Lowry et al. (15) with bovine serum albumin as standard.

Chemicals. TC and TUDC were donated by Tokyo Tanabe Co. (Tokyo, Japan). These bile acids were more than 98% pure when examined by HPLC. Purified egg yolk lecithin was a gift from S. Urano (Isotope Laboratory, Tokyo Metropolitan Institute of Gerontology). 5′-AMP, NAD, and 3α-hydroxysteroid dehydrogenase were purchased from Sigma Chemical Co. (St. Louis, Missouri). All other chemicals were obtained from various sources as analytical grade reagents.

RESULTS

Figure 1 shows the effect of bile acids on the solubilization of 5′-nucleotidase activity from liver plasma membrane of the rat. In the absence of bile acids, a negligible amount of 5′-nucleotidase activity was released into the soluble fraction from the membrane. This result suggested that mechanically disrupted membrane fragments did not pass through the 0.22 μm Millex-GV filter. When the membranes were incubated with various concentrations of bile acids, the release of 5′-nucleotidase activity from the membrane was dependent upon the bile acid concentration. The difference between TUDC and TC's ability to solubilize the enzyme from the membrane was remarkable at higher concentrations (20 and 40 mM). At 40 mM, TC released about 10 times more 5′-nucleotidase activity into the soluble fraction than TUDC.

Figure 2 shows the solubilization of 5′-nucleotidase activity from the membrane by various combinations of bile acid, lecithin and cholesterol. When the membrane was incubated with mixed micelles of TUDC-lecithin (40 and 8 mM), 10 times more 5′-nucleotidase activity was released than with 40 mM TUDC alone, while lecithin alone did not release any activity into the soluble fraction. Mixed micelles of TUDC-lecithin-cholesterol (40, 8 and 0.4 mM) had the same effect as the mixed micelles of TUDC-lecithin. When the membrane was incubated with TC-lecithin (40 and 8 mM) or TC-lecithin-cholesterol (40, 8 and 0.4 mM), these mixed micelles had the same effect as 40 mM TC alone in releasing the activity of the enzyme from the membranes.

In the case of TC, 40 mM TC alone already released the maximal enzyme activity from the membrane. But when lecithin was added to 20 mM TC, the mixed micelles released twice as much 5′-nucleotidase activity as 20 mM TC alone (Fig. 3). The effect of lecithin on the solubilization of 5′-nucleotidase by different concentrations of bile acids is shown in Figure 3. The difference between TUDC alone and TC alone in the ability to release the enzyme

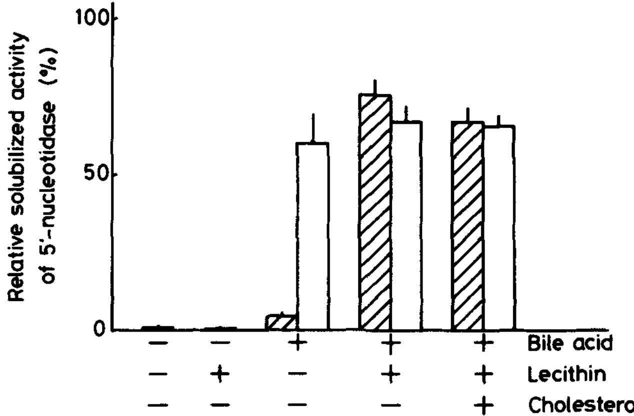

FIG. 2. Solubilization of 5′-nucleotidase from the liver plasma membrane of the rat by bile acids, bile acid-lecithin and bile acid-lecithin-cholesterol. Liver plasma membranes were incubated with mixtures of various combination of 40 mM bile acids (TUDC or TC), 8 mM lecithin and 0.4 mM cholesterol. The relative solubilized activity of 5′-nucleotidase was determined by the method described in the text. Values are means of three or four experiments. Vertical bar expresses S.D.; hatched columns, TUDC; open columns, TC.

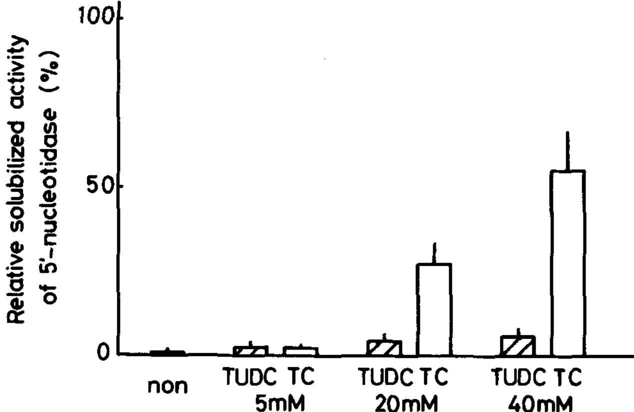

FIG. 1. Solubilization of 5′-nucleotidase from the liver plasma membrane of the rat by bile acids. Liver plasma membranes were incubated with various concentrations of bile acids (TUDC or TC). The relative solubilized activity of 5′-nucleotidase was determined by the method described in the text. Values are the means of three or four experiments; vertical bar expresses S.D.

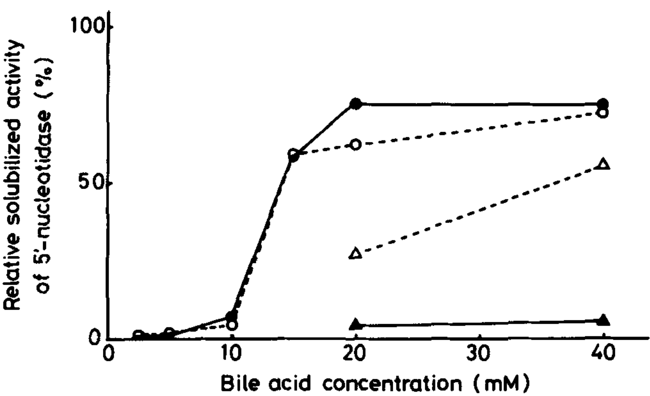

FIG. 3. Effect of lecithin on the solubilization of 5′-nucleotidase from rat liver plasma membrane by different concentrations of bile acids. Liver plasma membranes were incubated with various concentrations of bile acids containing either 8 mM egg yolk lecithin or none. The relative solubilized activity of 5′-nucleotidase was determined by the method described in the text. Values are means of four experiments. ●, TUDC + lecithin; ○, TC + lecithin; ▲, TUDC; △, TC.

disappeared with the addition of lecithin at the concentrations of bile acids tested. At lower concentrations of the bile acids (2.5 and 5 mM), 8 mM lecithin did not show any effect on the solubilization. At bile acid concentrations higher than 10 mM, lecithin had a pronounced effect on the solubilization by both bile acids.

When various concentrations of lecithin were added to 40 mM TUDC, the solubilization of 5′-nucleotidase increased in a concentration-dependent manner up to 2 mM (Fig. 4). Thereafter, the effect of lecithin remained steady.

To compare bile with bile acid-lecithin mixtures in the solubilization of 5′-nucleotidase from rat liver plasma membrane, TUDC bile and TC bile were used. When the membranes were incubated with these biles, the activities of 5′-nucleotidase in the soluble fractions were practically the same (Table 1).

DISCUSSION

In the present in vitro study, TUDC released less 5′-nucleotidase from the liver plasma membranes of the rat than TC. This is consistent with previous reports (4–7). Hatoff and Hardison (4) showed that TUDC was less effective than TC in the release of enzymes from liver plasma membrane both in vivo and in vitro. Barnwell et al. (5,6), using isolated perfused rat livers, and Kitani et al. (7), using bile fistula rats, reported similar observations. In the present study, however, mixed micelles of bile acids and lecithin did not reveal any difference between the bile acid species. Furthermore, the bile obtained from rats infused with TC or TUDC did not reveal any difference in their ability to solubilize 5′-nucleotidase. These findings suggest that there is no specific factor in bile that discriminates between the mixed micelles of native bile and synthetic mixed micelles in respect to the solubilization of 5′-nucleotidase. It cannot be absolutely stated that minor bile acids in TUDC bile are not the cause for its conspicuous ability to solubilize the membrane-bound proteins. But this hypothesis seems unlikely because when ursodeoxycholate was infused into rats, the main metabolite was TUDC and the second metabolite was a small quantity of β-muricholate, which is considered to have little surface activity or detergent effect (16–18).

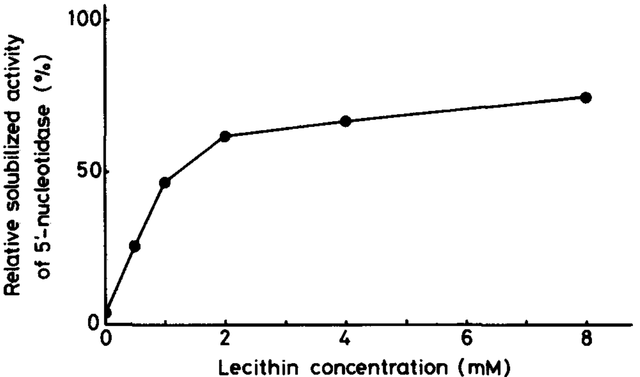

FIG. 4. The effect of different concentrations of lecithin on 40 mM TUDC's solubilization of 5′-nucleotidase from the liver plasma membrane of the rat. The liver plasma membranes were incubated with 40 mM TUDC containing various amounts of lecithin. The relative solubilized activity of 5′-nucleotidase was determined by the method described in the text. Values are means of three experiments.

TABLE 1

Solubilization of 5′-Nucleotidase from the Liver Plasma Membrane of Rat by Biles

Bile	Relative solubilized activity of 5′-nucleotidase (%)
TUDC bile	60.6 ± 3.9
TC bile	57.2 ± 2.7

Values are the means of four experiments ± S.D.

Recently Lowe et al. (19), using a perfusion of isolated rat liver, reported that mixed micelles cannot be formed inside the cell or during passage of bile salts through the membrane. They suggested that the formation of mixed micelles occurs in the bile canaliculus lumen. Their report prompts the speculation that the difference between the in vivo and in vitro effect of bile acids on solubilizing the enzyme from the membrane is that the release of the enzyme occurs soon after bile acid is secreted into the bile canaliculus and before the bile acid forms mixed micelles with the phospholipid. Once mixed micelles are formed, it might be difficult for them to interact with the membrane. Concerning this, Wilson and Dietchy (20), using rat intestine, reported that mixed micelles of bile acid and lecithin were much larger than single micelles of bile acid alone, so that the mixed micelles were more difficult to diffuse through the unstirred water layer that exists at the surface of membranes.

In the present study, the mixed micelles of bile acid-lecithin were more effective solubilizers than single micelles of bile acid alone. Nevertheless, Coleman et al. (21), using rabbit bile and glycodeoxycholate, reported that the release of hemoglobin and acetylcholinesterase from human erythrocytes was greatly reduced by the addition of lecithin. We cannot directly compare these results, since there are many differences in the experimental conditions.

Recently 5′-nucleotidase was purified from rat and mouse liver by several investigators (22–24). Widnell and Unkeless (22) and Nakamura (24) reported that partially purified 5′-nucleotidase contained phospholipids. They reported that 5′-nucleotidase interacted with both sphingomyelin and lecithin, which influenced the stability of the enzyme and the energy of activation, respectively (25). Furthermore, the partially purified enzyme easily reassociated with phospholipid vesicles by treatment with bile acid. Considering these results, the mixed micelles of bile acid-lecithin may include 5′-nucleotidase, released from the membrane by bile acid treatment.

In conclusion, in vitro, single micelles of TUDC or TC released 5′-nucleotidase from rat liver plasma membrane in a characteristic species-dependent manner. TUDC produced less solubilization than TC. These observations are comparable to similar studies in vivo. The differences in the solubilization by bile acids disappeared when the bile acids were mixed with lecithin. Similarly, there was no bile acid species difference for TUDC bile and TC bile in the solubilization of 5′-nucleotidase. These observations suggest that the interactions of bile acid and the membrane are greatly affected by lecithin.

ACKNOWLEDGMENTS

J. Ek reviewed the manuscript.

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Asymmetric Distribution of Arachidonic and n-3 Polyunsaturated Fatty Acids in Rat Liver Microsomal Membranes Under a Fat-Free Diet

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Rats adapted to a corn oil or a fish oil diet were fed a fat-free diet, and changes in phospholipid polyunsaturated fatty acids (PUFA) in the inner and outer leaflets of liver microsomal membranes were followed for 18 wk. In rats previously adapted to a corn oil diet, arachidonic acid in phosphatidylcholine and phosphatidylethanolamine in the inner and outer leaflets did not decrease quickly; rather, linoleic acid decreased more than arachidonic acid for the first three weeks of feeding the fat-free diet. Even at 18 wk, 40–50% of the beginning arachidonic acid levels were still retained. In contrast, in rats previously adapted to a fish oil diet, the n-3 PUFA were quickly decreased by the fat-free diet to only 10–30% at 18 wk. Due to the appearance and increase of n-9 eicosatrienoic acid in the replacement of the n-3 and n-6 PUFA, total PUFA did not decrease in the inner and outer phosphatidylcholine in either group, but decreased somewhat in the phosphatidylethanolamine due to the insufficient increase of the n-9. On the other hand, the overall degrees of unsaturation in phosphatidylcholine fatty acids were always higher in the outer than in the inner leaflets and were not altered by feeding the fat-free diet even for 18 wk. Thus, the results appear to reveal the physiological importance of unsaturation ratio of fatty acids and the necessity of arachidonic acid in each membrane leaflet.
Lipids 22, 45–50 (1987).

We previously reported that n-3 polyunsaturated fatty acids (PUFA) compete with arachidonic acid for the C-2 position of phospholipids of rat liver, plasma and heart (1). When rats adapted to a fat-free diet were fed a corn oil diet, endogenous n-9 eicosatrienoic acid (the major PUFA) at the C-2 positions of both phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was quickly substituted by arachidonic acid in liver, plasma and platelets (2). Comparably, with a fish oil diet, the n-9 was quickly substituted by n-3 PUFA. In both cases, the n-9 almost disappeared in six days. On the other hand, when the dietary process was reversed, arachidonic acid in both the phospholipid classes decreased slowly, whereas the n-3 quickly decreased in the platelets and the liver mitochondria and microsomes (2). These results may reveal the physiological significance of arachidonic acid in membrane phospholipids. On the other hand, the existence of phospholipid asymmetries in biological membranes has been demonstrated, and the membrane asymmetry would play an important role of physiological significance on membrane function (3–12). In the present experiment, the effects of the fat-free diet feeding on asymmetric distribution of PC and PE PUFA in the inner and outer monolayers of the microsomal membranes of rat liver have been investigated.

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MATERIALS AND METHODS

Animals. Male Wistar rats, 4 weeks old, were fed a 5% corn oil or 5% fish oil diet for 2 wk and then a fat-free diet for indicated periods. The fat-free diet contained 68.5% sucrose, 18% casein, 9.5% cellulose, 4% salt mixture (13), 0.1% choline chloride and vitamins (13). The amount of 5% sucrose was substituted by fat in the 5% fat diet. The fatty acid compositions of corn oil were 8.5% palmitic, 3.8% stearic, 40.1% oleic, 46.1% linoleic and 1.5% other minors. Those of fish oil (from pollack liver) were 24.3% palmitic, 6.9% palmitoleic, 4.3% stearic, 14.8% oleic, 0.7% linoleic, 4.0% arachidonic, 10.8% eicosapentaenoic, 25.6% docosahexaenoic and other minors. The rats were maintained under an automatic lighting schedule at 24 C and were given water and food ad libitum.

Preparation of microsomes. The animals were killed by decapitation at 9–10 a.m. on the days indicated in figures and tables. The livers were quickly removed to take microsomes. Liver homogenate was prepared in 3 vol of 0.25 M sucrose using a Teflon-glass homogenizer and centrifuged at 16,000 × g for 10 min. The supernatant was again centrifuged at 105,000 × g for 45 min (Spinco Model L-5 50, Beckman, Palo Alto, California). The 105,000 × g pellets were used as microsomal fractions.

Incubation with phospholipase C and lipid extraction. Microsomes (10–15 mg) were incubated with 2–6 U phospholipase C from *Clostridium welchii* (Type X, Sigma Chemical Co., St. Louis, Missouri) in 2 ml of 0.25 M sucrose, 1 mM $CaCl_2$ and 10 mM Tris-HCl, pH 7.5, at 25 C for 30 min, essentially according to the method of Higgins and Dawson (10). After incubation, an aliquot of the microsomes was taken to be measured for mannose-6-phosphatase, as described below. The incubation mixture with phospholipase C was extracted with 20 ml of chloroform/methanol (2:1, v/v), and lipids were contained in the upper phase. The lipid extract was dissolved in chloroform/methanol (1:1, v/v), and an aliquot was used for separation of lipid classes. PC was separated on thin layers of silica gel (Merck, Darmstadt, Federal Republic of Germany), using a one-dimensional system (chloroform/methanol/water, 65:25:4, v/v/v) and estimated as the amount in inner membranes. Lipid-containing spots were detected by iodine vapor, scraped into test tubes and digested with perchloric acid for 30 min at 230 C to measure phosphorus. Water was added and the tubes were centrifuged to remove the silica gel. Phosphorus was determined on the supernatant by the method of Bartlett (14). An aliquot of lipids was used for analysis of fatty acids. Fatty acids were analyzed by gas chromatography as described previously (2). The amounts of PC fatty acids in the outer monolayer of the membranes were calculated by subtracting the amount of inner fatty acids from total PC fatty acids, which were similarly measured using intact microsomes.

Labeling with trinitrobenzenesulfonate (TNBS). PE in the cytosolic site of membranes was labeled with TNBS

(11). Microsomes (12 mg of protein) were incubated at 25 C in 6 ml of a medium containing 10 mM Tris-HCl (pH 7.7), 0.25 M sucrose and 1 mM TNBS for 20 min (when not otherwise indicated). After incubation, the reaction was stopped by acidification of the medium to pH 6.8 and centrifuged for 20 min at 40,000 × g. The supernatant fluids were discarded, and the pellets were washed twice with 10 ml of the suspending medium without the probes. Lipids were extracted from the layers with chloroform/methanol (2:1, v/v) and the two forms of PE (with and without labeling with TNBS) were separated on thin layers of silica gel using a solvent of chloroform/methanol/water (65:25:4, v/v/v). Phosphorus and fatty acids were determined as described above.

Measurement of leakage of microsomal content. To examine leakage of microsomal content during the incubation of microsomes with phospholipase C, the leak of mannose-6-phosphatase activity was measured (15). For assay of the mannose-6-phosphatase activity, microsomes were incubated with 4 mM mannose-6-phosphate, 1 mM EDTA, 60 mM malate buffer, pH 6.5 and 0.25 M sucrose at 37 C for 15 min. The incubation was stopped by adding 1 ml of 10% TCA, and the phosphorus amount in the supernatant was measured by the method of Bartlett (14). Total mannose-6-phosphatase activity was similarly measured using disrupted microsomes. The disrupted microsomes were prepared to 0 C by supplementing 9 vol of intact microsomes (1 to 2 mg of protein/ml) with 1 vol of 4% sodium taurocholate, pH 7.6. The taurocholate-dispersed microsomes were kept on ice at least 30 min before assays were performed (16).

RESULTS AND DISCUSSION

Treatment of liver microsomes by phospholipase C. Hydrolysis of microsomal PC by increasing concentrations of phospholipase C proceeded until ca. 70–75% of PC phosphate was removed after 30 min of incubation. The hydrolysis was almost constant in the range of 1–3 U phospholipase C/ml (Fig. 1). The amount of PC hydrolyzed increased linearly with microsomal concentrations (5–20 mg), but the percentage remained constant (data not shown). Although hydrolysis of PC was tried by several concentrations of phospholipase C (1–3 U/ml) for each sample, the experimental conditions described above were available for all samples during the long-term feeding of the fat-free diet. The microsomes incubated with 1.5 U phospholipase C/ml were used for the determination of mannose-6-phosphatase and the analysis of PC fatty acids.

Leakage of microsomal content. To determine if microsomal vesicles remained intact under the contributions of incubation with phospholipase C, mannose-6-phosphatase latency was determined at the same protein concentrations as a quantitative index of microsomal integrity. Less than 6% of mannose-6-phosphatase leaked into the incubation medium, indicating that the microsomal permeability barrier remained almost intact.

Labeling of outer PE with TNBS. TNBS labeling of microsomes converted ca. 30% of PE to trinitrophenyl PE, and this value did not increase when the incubation time was increased to 60 min. As shown in Figure 2, the reaction reaches a plateau at 0.5–2 mM TNBS. TNBS labeling of microsomes was conducted in a medium of 1 mM TNBS for 20 min at 25 C, as the conditions were available for all samples under the fat-free diet for 18 wk.

Phospholipid fatty acid composition in inner and outer leaflets of rat liver microsomal membranes. The effects

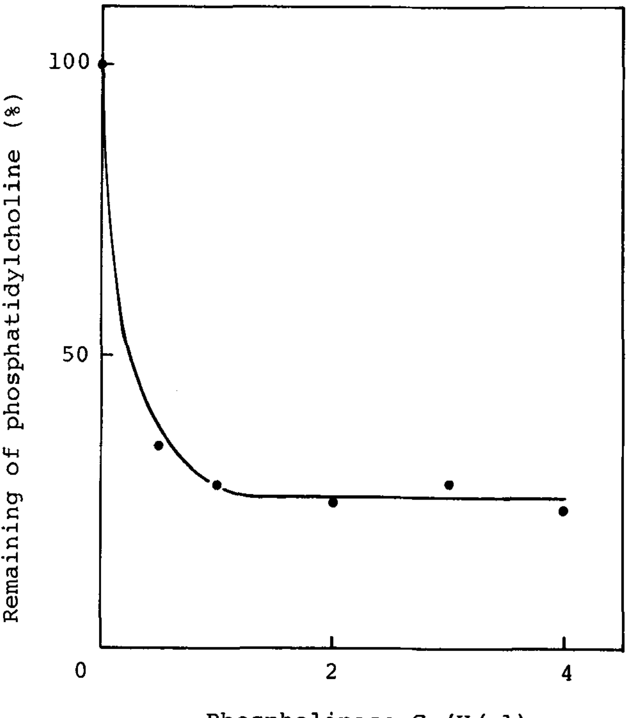

FIG. 1. Hydrolysis of microsomal phosphatidylcholine by phospholipase C. Liver microsomes (10–15 mg protein) were incubated with 2–6 U phospholipase C from *Clostridium welchii* in 2 ml for 30 min at 25 C. The lipids were extracted and remaining phosphatidylcholine was separated on thin layers of silica gel.

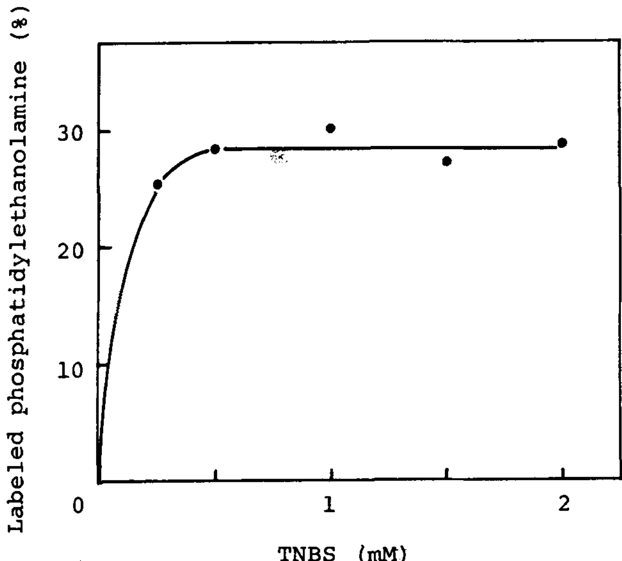

FIG. 2. The extent of reaction of microsomal phosphatidylethanolamine with trinitrobenzenesulfonate (TNBS). Rat liver microsomes (12 mg protein) were incubated with TNBS for 20 min at 25 C. The lipids were extracted and then the two forms of phosphatidylethanolamine (with and without labeling with TNBS) were separated on thin layers of silica gel.

of dietary fat manipulation on distribution of phospholipid fatty acids in the inner and outer leaflets of rat liver microsomal membranes were investigated. The fatty acid compositions in PC and PE of the inner and outer leaflets are shown in Tables 1 and 2. The results in rats adapted to corn or fish oil diets and in those subsequently fed a fat-free diet for 18 wk were compared. The predominant species of fatty acids are the same among the inner and outer PC and PE, except that in the animals adapted to the corn oil diet, linoleic acid is one of the predominant species in the outer PC, but not in the inner PC or the inner and outer PE. The overall degree of unsaturation was used as a parameter for comparing the fatty acid distribution as an "unsaturation ratio," which is defined as the total amount of unsaturated fatty acids divided by the total amount of saturated ones. The unsaturation ratios in the inner and outer PC and PE were unchanged by the long-term feeding of the fat-free diet, as shown in Tables 1 and 2. In PC, however, the unsaturation ratios were significantly higher in the outer than the inner leaflet

TABLE 1

Compositions of Fatty Acids of Phosphatidylcholine in the Inner and Outer Microsomal Membranes in Rat Liver

	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:5	22:6	Unsaturation ratio
Corn oil-adapted rats										
Outer membrane										
0 wk fat-free diet	6.84	3.30	5.26	4.17	4.47	—	7.45	0.10	0.87	1.67 ± 0.08
18 wk fat-free diet	7.22	3.65	5.46	8.23	0.60	6.85	3.17	—	0.41	1.80 ± 0.10
Inner membrane										
0 wk fat-free diet	2.79	1.23	2.64	1.78	0.94	—	2.64	—	0.44	1.30 ± 0.04*
18 wk fat-free diet	3.62	1.63	2.91	2.96	0.40	1.97	1.09	—	0.44	1.30 ± 0.05*
Fish oil-adapted rats										
Outer membrane										
0 wk fat-free diet	7.05	2.99	3.84	5.34	1.49	—	3.33	4.11	3.29	1.88 ± 0.06
18 wk fat-free diet	7.93	4.39	6.76	8.29	0.73	8.34	2.52	0.16	0.75	1.71 ± 0.06
Inner membrane										
0 wk fat-free diet	3.33	1.35	2.42	2.06	0.42	—	1.41	0.60	1.48	1.28 ± 0.03**
18 wk fat-free diet	2.86	1.40	2.93	2.45	0.39	2.16	0.86	0.09	0.62	1.38 ± 0.03**

Rats adapted to a corn or fish oil diet were fed a fat-free diet for 18 wk. Fatty acid compositions were compared between, at the beginning and the end of the fat-free diet feeding. To avoid complication, only mean values (μg/mg protein) are shown for the amounts of fatty acids in the inner and outer leaflets of liver microsomal membranes. Unsaturation ratios are defined as the total amount of unsaturated fatty acids divided by the total amount of saturated ones. Mean or mean ± SE (n = 3-5). Significantly different from the corresponding outer membrane. *, $P < 0.01$, **, $P < 0.001$ (by Student's t-test).

TABLE 2

Compositions of Fatty Acids of Phosphatidylethanolamine in the Inner and Outer Microsomal Membranes in Rat

	16:0	16:1	18:0	18:1	18:2	20:3	20:4	20:5	22:6	Unsaturation ratio
Corn oil-adapted rats										
Outer membrane										
0 wk fat-free diet	2.65	1.60	1.53	1.82	0.58	—	1.22	0.02	0.43	1.36 ± 0.05
18 wk fat-free diet	2.67	1.39	0.82	2.52	0.40	0.50	0.54	—	0.31	1.51 ± 0.13
Inner membrane										
0 wk fat-free diet	3.32	1.73	3.09	2.14	0.73	—	3.65	0.25	1.15	1.51 ± 0.03
18 wk fat-free diet	2.96	1.60	2.22	2.22	0.44	0.88	1.81	—	0.64	1.45 ± 0.03
Fish oil-adapted rats										
Outer membrane										
0 wk fat-free diet	3.32	2.36	1.40	1.52	0.32	—	0.54	0.36	1.33	1.37 ± 0.02
18 wk fat-free diet	2.61	1.41	0.99	2.19	0.24	0.44	0.54	-	0.41	1.45 ± 0.03
Inner membrane										
0 wk fat-free diet	4.11	2.39	2.44	1.80	0.36	—	1.44	0.95	3.00	1.52 ± 0.06
18 wk fat-free diet	3.24	1.50	1.34	2.16	0.22	1.17	1.61	0.10	0.84	1.66 ± 0.15

Rats adapted to a corn or fish oil diet were fed a fat-free diet for 18 wk. To avoid complication, only mean values (μg/mg protein) are shown for the amounts of fatty acids in the inner and outer leaflets of liver microsomal membranes. Unsaturation ratios are defined as the total amount of unsaturated fatty acids divided by total amount of saturated ones. No significant difference was found among the results. Mean or Mean ± SE (n = 3-5).

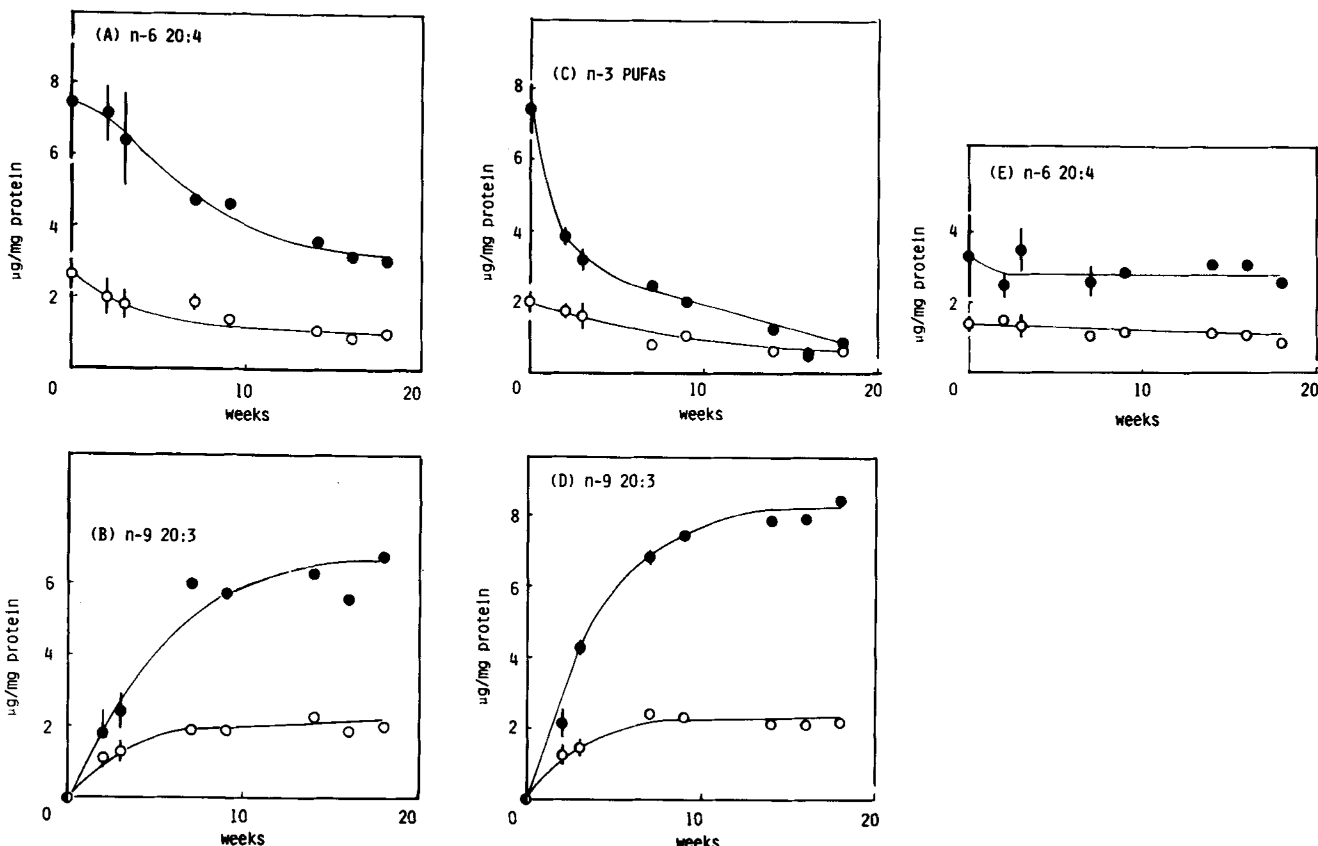

FIG. 3. Effects of feeding a fat-free diet on polyunsaturated fatty acids (PUFA) with more than three double bonds in phosphatidylcholine in the inner and outer leaflets of liver microsomal membranes. In rats previously adapted to corn oil or fish oil diets and then fed a fat-free diet, the levels of PUFA were followed for 18 wk. White and black circles show the PUFA levels in the inner and outer leaflets, respectively. A, B: rats adapted to the corn oil diet; C, D, E: rats adapted to the fish oil diet. Mean or mean ± SE (n = 3–5).

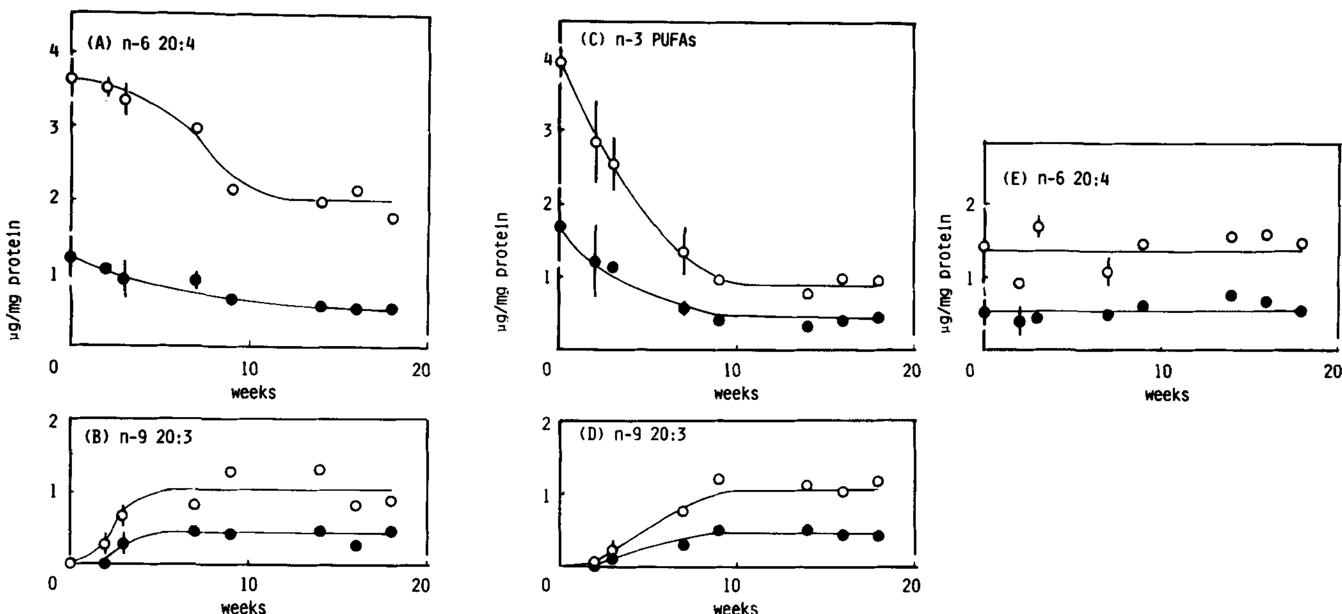

FIG. 4. Effects of feeding a fat-free diet on polyunsaturated fatty acids (PUFA) with more than three double bonds in phosphatidylethanolamine in the inner and outer leaflets of liver microsomal membranes. Levels of PUFA in the inner and outer phosphatidylethanolamine of the liver microsomal membranes in the same animals are shown in Fig. 3. White and black circles show the PUFA levels in the inner and outer leaflets, respectively. A, B: rats adapted to the corn oil diet; C, D, E: rats adapted to the fish oil diet. Mean or Mean ± SE (n = 3–5).

in both the corn oil and the fish oil group, whereas the ratios in PE were similar in the inner and outer leaflets.

Changes in phospholipid PUFA under a fat-free diet. Figure 3 shows time courses of PC PUFA levels in the inner and outer leaflets of liver microsomal membranes of rats previously adapted to corn or fish oil diets and then fed the fat-free diet. The time courses of PUFA in the inner and outer PE are shown in Figure 4.

In rats adapted to a corn oil diet and then fed a fat-free diet, arachidonic acid in PC of the outer leaflets decreased only slightly in 2 wk and to 87% in 3 wk, while arachidonic acid in the inner PC decreased to 68% in 3 wk. In the outer and inner PC, linoleic acid decreased more quickly than arachidonic acid for the first three weeks of the fat-free diet. Linoleic acid decreased to only one-third in 3 wk. This appears to be an arachidonic acid–saving mechanism. The inner and outer PE arachidonic acid likewise did not decrease for the first three weeks. Even after 18 wk of the fat-free diet, arachidonic acid still remained at 40–50% of the beginning levels in the inner and outer PC and PE. The n-9 eicosatrienoic acid appeared in 2 wk and increased enough to replace the decrease of arachidonic acid in the inner and outer PC in 18 wk. Due to the increase of the n-9, the beginning levels of total PUFA with more than three double bonds in PC were kept even for 18 wk. In the inner and outer PE, however, n-9 eicosatrienoic acid increased to only half the level of the decrease of arachidonic acid. Therefore, the total PUFA levels in PE decreased to 80% of the beginning level, due to the insufficient increase of the n-9.

In rats adapted to a fish oil diet and then fed a fat-free diet, the n-3 PUFA in the outer PC was decreased to half the level of the beginning in 2 wk and to only 12% in 18 wk. In the inner PC, n-3 PUFA decreased to 85% of the beginning level in 2 wk and to 33% in 18 wk. The n-3 PUFA decreased to 72% in the inner and outer PE in 2 wk and to 25% in 18 wk. However, the levels of arachidonic acid in the inner and outer PC and also PE were not much changed during the long-term feeding of the fat-free diet. With a fat-free diet, the n-3 PUFA in the inner and outer PC and PE in rats previously adapted to a fish oil diet were quickly and greatly decreased compared to the

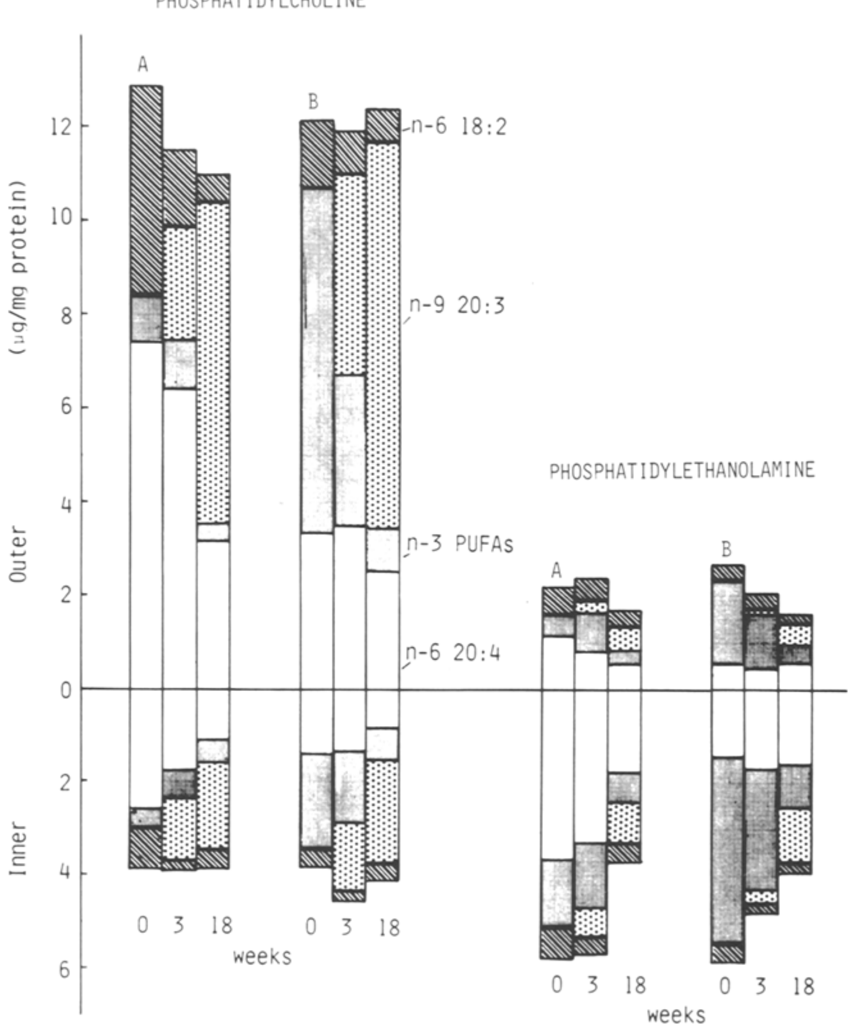

FIG. 5. Effects of feeding the fat-free diet for 3 or 18 wk on total polyunsaturated fatty acids (PUFA) of phospholipids in inner and outer leaflets of liver microsomal membranes. A, rats adapted to the corn oil diet; B, rats adapted to the fish oil diet. Mean (n = 3–5).

arachidonic acid levels of the rats previously adapted to a corn oil diet. In the replacement of n-3 PUFA, n-9 eicosatrienoic acid appeared in the inner and outer PC in 2 wk and slightly in the PE in 3 wk of the fat-free diet. The appearance of n-9 eicosatrienoic acid was delayed in the PE, especially in the fish oil group, as compared to the corn oil group. Moreover, the n-9 did not increase to the same degree as the decrease of the n-3 PUFA in the inner and outer PE.

Figure 5 shows total PUFA with more than two double bonds in PC and PE in the inner and outer leaflets of liver microsomal membranes. Considerable amounts of arachidonic acid still remained in each leaflet, and the amounts were similar between the animals adapted to a fish oil diet and those fed the fat-free diet, even for 18 wk. Therefore, these arachidonic acid levels appear to be the minimum for the physiological membrane functions. Total arachidonic acid levels in PC plus PE were higher in the outer than in the inner leaflet. Further, although linoleic acid decreased more quickly than arachidonic acid in the outer PC, a small amount of linoleic acid was retained in each leaflet even after the long-term feeding of the fat-free diet. The linoleic acid itself also may be retained for a physiological role.

Spector and Yorek (17) reported in a review that membrane fatty acid and phospholipid compositions can be modified in many different kinds of intact mammalian cells, and the modifications are extensive enough to alter membrane fluidity and affect a number of cellular functions. As a result of the present experiment, it is suggested that the unsaturated fatty acid–generating system has a tendency to compensate for the lack of essential fatty acid intake for the regulation of homeostasis in membrane fluidity and some cellular functions.

On the other hand, the endoplasmic reticulum is the major site of phospholipid biosynthesis. Ballas and Bell (12) reported that the biosynthesis of phospholipids and triglycerides occurs asymmetrically on the cytoplasmic surface of the endoplasmic reticulum. We have found that the degree of unsaturation in PC fatty acids was always higher in the outer than in the inner leaflet of the membrane bilayer. In addition, the unsaturation degrees in both the inner and outer PC and PE were not altered even by long-term feeding of the fat-free diet. The asymmetrical distribution of unsaturated fatty acids in the outer leaflet may be related to the similar distribution of the glycerolipid-generating system. The specific saving of arachidonic acid and the retaining of unsaturation degrees of fatty acids in the inner and outer leaflets of membrane bilayers may have an important relationship to membrane functions.

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Lipids from *Plasmodium vinckei*-Infected Erythrocytes and Their Susceptibility to Oxidative Damage

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The constituent lipids of plasma and red blood cells (RBC) from mice late in infection with the malarial parasite *Plasmodium vinckei* were analyzed and compared with those obtained from uninfected animals. On a dry weight basis, the total extractable lipids of RBC increased threefold during infection, while those of the plasma did not change significantly. In general, changes in individual plasma lipid constituents paralleled those found in RBC of infected mice but were of smaller magnitude. While the increase in the total lipids of parasitized RBC was largely attributable to an increase of more than fourfold in total phospholipids, a significant increase in neutral lipids was also observed. Phosphatidylcholine and phosphatidylethanolamine were the major phospholipids present within RBC, and their total and relative concentrations increased as a result of the infection. A parallel increase occurred in the ratio of unsaturated to saturated fatty acids in the parasitized RBC phospholipids. Infection was also associated with decreases in the relative amount of cholesterol present in RBC and in the ratio of cholesterol to phospholipid. Consistent with this, the fluorescence polarization of 1[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene within parasitized RBC plasma membranes was decreased in comparison with its value in noninfected RBC, indicating that malarial infection decreases the "order" of membrane lipids. These modifications, in conjunction with the increased levels of vitamin E and malonyldialdehyde reported elsewhere, are important determinants of the susceptibility of the different membrane compartments within infected RBC to peroxidative damage.
Lipids 22, 51-57 (1987).

Protozoan parasites of the genus *Plasmodium,* which cause malaria, undergo repetitive cycles of asexual multiplication in appropriate vertebrate hosts. Replication occurs initially within liver parenchymal cells and, subsequently, within circulating red blood cells (RBC) following entry of the merozoite stage by invagination of the host cell membrane (1). This process gives rise to the parasitophorous vacuole, a membrane structure of RBC origin that surrounds the intracellular parasites. The cytosolic compartments of intraerythrocytic parasites therefore are separated from the blood plasma by three membranes: the plasma membrane of the RBC, the boundary membrane of the parasitophorous vacuole and the parasite plasma membrane (1). During its intraerythrocytic development, the parasite participates in dynamic exchanges of a diversity of metabolites with the blood plasma (2), which may be facilitated by changes in the structure and composition of the appropriate intervening membranes (2,3). In general, total lipids increase in malaria-infected RBC, although the extent of this increase varies between species (2-4). There is, in addition, no common pattern linking the changes that occur in the relative amounts of the major fatty acids of neutral lipids and phospholipids in malaria-infected RBCs from ducks, mice and monkeys (3,5,6).

Changes in the lipid composition and degree of saturation of the constituent fatty acids of membrane lipids are generally accompanied by alterations in the physicochemical properties of the membrane. Such changes may influence the activities of membrane-bound enzymes, membrane "fluidity" and the susceptibility of membrane components to oxidative damage (7-10). The latter may be important in the development of new antimalarial drugs, since work from this laboratory has shown that, in vitro and in vivo, murine and human malarial parasites are destroyed within intact host RBC membranes by oxygen radical-producing drugs (11-14). This observation implies a difference in the susceptibility of erythrocytic and parasitic membranes to oxidative damage. Vitamin E is the most powerful lipid-soluble antioxidant, and the large increase in its content that occurs within *P. vinckei*-infected RBC appears to be associated with the host, rather than the parasite, plasma membrane (15). One implication of this uneven distribution is that the membranes in closest proximity to the parasite might contribute selectively to the increase in spontaneous lipid peroxidation that occurs in response to increasing parasite load and maturation (15). However, factors such as differences in the amount and composition of constituent lipids are also likely to be critical in determining the relative susceptibilities of the different membranes to peroxidative damage (16). Thus, to attempt to explain the increased sensitivity of membranes from *P. vinckei*-infected RBC to peroxidative attack (11-15), it was necessary to know the lipid composition of the infected RBC and its uninfected counterpart. However, this has never been analyzed in *P. vinckei*-infected mice, and extrapolation from data obtained with other species is not justifiable because of the variability mentioned above (2-6).

MATERIALS AND METHODS

Plasmodium vinckei subsp. *vinckei,* strain V52, originally obtained from F.E.G. Cox, King's College, London, was used for all experiments. Mice were held in a natural day-night cycle at 22 C and fed with a standard laboratory rat and mouse ration (Barastoc Feeds, Murrumburah, Australia). Male CBA/CaH mice, 6-10 wk old, were infected by intraperitoneal injection of 10^6 parasitized RBC, and the extent of parasitemia was assessed by counting, on a blood smear, 200-300 cells stained with Harleco's Diff-Quik (American Hospital Supply, Sydney, Australia). Blood from control or infected animals was collected under anesthesia from the subclavian artery, heparinized (20 units/ml blood) and separated into plasma and cells by centrifugation at 2200 × g for 10 min at 4 C. Cells were resuspended in phosphate-buffered saline to 10% hematocrit and passed through a cellulose powder CF-11

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column (Whatman, Maidstone, United Kingdom) to remove leukocytes before being washed twice with the same buffer. Packed cell volume was determined after the final wash by further centrifugation of the RBC under similar conditions to those described above.

Lipids (total lipids) were extracted from RBCs obtained from the pooled blood of 12–15 control or 15–20 heavily infected mice (parasitemias >80%), as described by Baech et al. (3) using chloroform and methanol containing 0.01% (w/v) butylated hydroxytoluene (Sigma, St. Louis, Missouri). Plasma lipids were extracted by the method of Sperry and Brand (17), applying their procedure 2 for purification of the lipid extracts. Values for "total lipids" were obtained gravimetrically after drying. If necessary, samples of these total lipids in chloroform/0.01% butylated hydroxytoluene were sealed under N_2 and stored at −60 C in the dark until required for further analysis. Samples of total lipids were separated into neutral lipids and phospholipids with silica Sep-Pak cartridges (Waters, Milford, Massachusetts) as described by Bitman et al. (18), and the eluates were dried with a rotary evaporator (Büchi, Flawil, Switzerland) and weighed. Samples of neutral lipids from the silica column were assayed as described for cholesterol (19) and triglycerides (20).

Quantitative analysis of individual phospholipid components in fractions obtained from the silica column was performed by separation on 20 × 20 cm preparative silica gel H TLC-plates (Merck, No. 5744, Darmstadt, West Germany). Aliquots from the phospholipid fraction, containing 2 mg lipid, were applied in 2-cm bands to the plates. A mixture of phospholipid standards (Sigma) containing, in 100 μg, equal amounts of lysolipid (LL), sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE) was also spotted on the left and right sides of the plates. Phospholipids were developed with chloroform/methanol/acetic acid/water (50:30:10:3, v/v/v/v) (21) and visualized by iodine vapor, and the areas of silica gel that corresponded to individual phospholipids were scraped from the plates. The silica was powdered and phospholipids were eluted sequentially in four separate steps, using different solvent mixtures and vigorous shaking in between with a Rotary Evapo-Mix (Buchler, Fort Lee, New Jersey) (21). Individual phospholipids were dried at 45 C under N_2, and an aliquot was transferred to an acid-washed glass tube for phosphorus analysis. Phosphorus determination was performed according to Chen et al. (22), with the following modification: 25 μl of concentrated sulfuric acid was added to the dried sample, which was then digested at 250 C for 30 min. Phosphate-free perchloric acid (35 μl) was added and the tubes again were incubated at 250 C until the black solution lost its color (about 2 hr). Tubes were cooled, 0.5 ml water and 0.6 ml of 0.4% (w/v) ammonium molybdate plus 0.1 ml of 10% (w/v) ascorbic acid were added and the samples were incubated at 45 C for 20 min. Color formation was quantitated by reading absorbance at 820 nm; the phosphorus content was determined by comparison with standards containing 0–1.6 μg phosphorus.

Aliquots (5–10 mg) of various purified lipids from RBCs and plasmas were taken up in 4 ml of 5% (w/v) KOH (in 50% ethanol, v/v), saponified under N_2 for 1 hr at 70 C and dried at 60 C in a rotary evaporator. Water (5 ml) was added and non-saponified material removed by extracting twice with an equal volume of freshly distilled petroleum ether and discarding the organic phase. The aqueous phase was acidified to pH 1 with 1 M HCl, and the fatty acids were extracted three times with 3 ml diethyl ether, dried over anhydrous Na_2SO_4 and taken to complete dryness in a rotary evaporator. They were then dissolved in 100–400 μl ethanol and analyzed by gas liquid chromatography (GLC).

GLC analyses of fatty acids were performed with a Pye Unicam (Cambridge, England) gas chromatograph series 204 equipped with a flame ionization detector and connected to a Spectra Physics (Santa Clara, California) SP 4100 computing integrator. The glass column (4 mm i.d. × 1 m), packed with pretested 5% DEGS-PS on Supelcoport 100/120 mesh (Supelco, Bellefonte, Pennsylvania), was run at an initial temperature of 200 C with N_2 as the carrier gas set at a flow rate of 55 ml/min. Upon sample injection, a temperature program was initiated that increased the temperature at a rate of 2 C/min to a final value of 225 C. The injection port and detector temperature were set to 210 C and 250 C, respectively. Relative distribution of the individual fatty acids was assessed by comparing the integrated areas under the peaks with those obtained from known amounts of external standards (Sigma).

Labeling of the RBC with 1[4-(trimethylamino)phenyl]-6-phenylhexa-1,3,5-triene (TMA-DPH; from Molecular Probes, Junction City, Oregon) was performed according to Kuhry et al. (23). Briefly, 20 μl of a 500 μM stock solution of TMA-DPH in dimethylformamide was added to 980 μl PBS with vigorous vortexing, and then 600 μl of this suspension was added to a fluorescence cuvette in the dark. RBC (3×10^7 in 100 μl) were added immediately followed by 2.3 ml PBS, and the whole was incubated at 25 C in the dark for 5 min. Maximum incorporation of TMA-DPH into the outer cell membrane is achieved after 1 min (23).

Fluorescence anisotropy measurements were performed at 25 C in a thermostated Perkin Elmer LS-5 Luminescence Spectrometer equipped with an electronically driven polarization unit. The sample was excited with vertically polarized light at 357 nm (slit width 5 nm), and the vertical (I_v) and horizontal (I_H) components of the fluorescence emission intensity were measured at 464 nm (slit width 10 nm). I_v was corrected for the response of the emission monochromator and photomultiplier tube system by multiplying I_v by the correction factor, G (where $G = I_v/I_H$, with horizontally polarized excitation light). The steady-state anisotropy (r_s) was determined as $r_s = (I_v - I_H)/(I_v + 2I_H)$. I_v and I_H were corrected for light scattering using unlabeled cells as described by Kuhry et al. (23).

Malonyldialdehyde was determined as described previously (24) with slight modification (13). Vitamin E was determined by a fluorimetric method (25).

Spectrophotometric grade solvents were used for the extraction and analysis of lipids. Petroleum ether (30–75 C), n-heptane and acetylacetone were redistilled before use.

RESULTS

The relative contributions of the major lipids to the total present in RBC from control CBA/CaH mice were PC (26%), PE (17%), cholesterol (15%) and SM (6%) (Table 1). Other phospholipids were detected in small amounts, as were traces of triglyceride. In control RBC,

TABLE 1

Relative Distribution of Lipid Classes and Major Lipid Types in Erythrocytes and Plasma of Control and *P. vinckei*-Infected Mice[a]

	Percentage of total lipids			
Lipid classes	Erythrocytes from control mice	Erythrocytes from infected mice	Plasma from control mice	Plasma from infected mice
Total lipids	100	100	100	100
Neutral lipids	21.2 ± 2.8	19.5 ± 2.2	54.6 ± 3.6	45.2 ± 5.0
Cholesterol	15.3 ± 1.4	9.8 ± 0.7[b]	16.5 ± 2.6	14.0 ± 1.0
Triglycerides	0.1 ± 0.2	6.6 ± 1.4[c]	13.4 ± 1.7	19.2 ± 2.9[c]
Phospholipids	60.0 ± 5.2	79.5 ± 6.7[c]	40.5 ± 5.5	49.8 ± 10.0
Phosphatidylethanolamine	17.3 ± 1.5	25.9 ± 1.5[c]	1.2 ± 0.2	1.9 ± 0.6
Phosphatidylserine / Phosphatidylinositol	1.4 ± 0.2	1.0 ± 0.1[b]	1.7 ± 0.7	2.9 ± 0.6
Phosphatidylcholine	26.0 ± 1.6	47.7 ± 3.9[c]	27.7 ± 4.0	32.5 ± 3.5
Sphingomyelin	5.7 ± 0.4	1.6 ± 0.4[b]	1.4 ± 0.3	1.5 ± 0.2
Lysolipids	1.9 ± 0.2	trace	4.3 ± 1.5	2.0 ± 0.6

[a]Percentages are calculated from results shown in Table 2. Values are mean ± S.D.
[b]Significantly lower ($P < 0.05$) than corresponding control value (Rank test).
[c]Significantly higher ($P < 0.05$) than corresponding control value.

neutral lipids and phospholipids made up 81% of the total lipid content, while in the parasitized RBC their contribution increased to 99% (Table 1). This result indicates that lipid components other than those selected for study in the present experiments, e.g., glycosphingolipids, may change their relative concentrations during the course of malarial infection, as reported by others (2).

The late stage of infection of mice with *P. vinckei* (>80% parasitemia) was characterized by a threefold increase in the amount of total lipids extractable from their RBC (Table 2), this being broadly similar to others' observations with different malaria species (2,4). This increase was associated with even larger changes (4-fold) in total phospholipids, which included marked elevations in PC (5.5-fold) and PE (4.5-fold), while the combined levels of PS and PI doubled. This significant increase in PC was not seen in *P. lophurae* and *P. berghei* infection (2,4). No significant changes were observed in the concentration of SM, in contrast to infections with other malaria species (2), while the amount of LL decreased in response to the infection. Neutral lipids were also elevated (2-fold), with the largest increase occurring in triglycerides. The doubling of the cholesterol content did not match the more striking increase in phospholipids, resulting in a significant decrease in the cholesterol-to-phospholipid ratio (Table 2). This is similar to previous observations with *P. knowlesi* and *P. lophurae*, but not with *P. berghei*, where no change was observed (2).

The relative amounts of the major lipids in plasma from control mice were PC (28%), cholesterol (17%), triglycerides (13%) and LL (4%) (Table 2). PE and SM were minor components, while unesterified fatty acids and cholesterol esters were not included in this analysis. Infection of mice with *P. vinckei* produced no significant change in plasma total lipid content (Table 2), although a substantial increase has been reported in *P. knowlesi* infection (4). Neutral lipid and cholesterol decreased in absolute amounts, in contrast to previous studies with *P. knowlesi*, *P. lophurae* and *P. berghei* (4). The plasma concentrations of total and individual phospholipids did not change significantly, with the single exception of LL, which decreased in parasitized mice (Table 2).

The fatty acids represented most prominently in lipids isolated from RBC and plasma from control and *P. vinckei*-infected mice were palmitic (16:0), stearic (18:0), oleic (16:1), linoleic (18:2) and arachidonic (20:4), although minor amounts of other fatty acids were also present (Tables 3 and 4). Phospholipids extracted from the RBC of infected mice contained relatively higher amounts of polyunsaturated fatty acids, while monounsaturated and saturated fatty acids were proportionately decreased (Table 5), contrasting with other reports (4). This increase in the degree of unsaturation of membrane phospholipids was due mainly to a significant increase in linoleic acid and decreases in palmitic and stearic acids in parasitized RBC (Table 3).

Malaria infection did not cause profound changes in the composition of fatty acids isolated from the phospholipids (Table 3) and neutral lipids (Table 4) of plasma. The only significant differences were a higher concentration of linoleic acid in the plasma phospholipids of infected mice (Table 3), reflecting the situation found in the RBC, and a significant decrease in arachidonic acid in plasma neutral lipids (Table 4).

Although most of the additional lipids in parasitized RBC seem to be associated with the parasite (3,26,27), at least part of the observed fluctuations might reflect changes in the lipid composition or organization of the RBC plasma membrane. Consistent with this suggestion, infected RBC had a decreased steady-state fluorescence anisotropy (r_s) of TMA-DPH (Fig. 1), a cationic fluorescent probe that remains localized in the plasma membrane of intact cells during the first 25 min of interaction (28,29).

In an attempt to assess the potential susceptibility of the membrane lipids of control and parasitized RBC to peroxidative damage, we compared the concentrations of vitamin E, on the one hand, and malonyldialdehyde produced spontaneously during in vitro incubation at 37 C,

TABLE 2

Weights of Lipid Classes and Major Lipid Types of the Erythrocytes and Plasma of Control and *P. vinckei*-Infected Mice[a]

Lipid classes	Erythrocytes from control mice	Erythrocytes from infected mice	Plasma from control mice	Plasma from infected mice
Total lipids	562 ± 17	1711 ± 164[c]	582 ± 65	480 ± 94[b]
Neutral lipids	119 ± 16	334 ± 38[c]	318 ± 21	217 ± 24[b]
Cholesterol	86 ± 8	167 ± 12[c]	96 ± 15	67 ± 5[b]
Triglycerides	1 ± 1	112 ± 24[c]	78 ± 8	92 ± 14[c]
Phospholipids	337 ± 29	1361 ± 115[c]	236 ± 32	239 ± 48
Phosphatidylethanolamine	97 ± 7	434 ± 25[c]	7 ± 1	9 ± 3
Phosphatidylserine / Phosphatidylinositol	8 ± 1	17 ± 2	10 ± 4	14 ± 3
Phosphatidylcholine	146 ± 9	816 ± 67[c]	161 ± 23	156 ± 17
Sphingomyelin	32 ± 2	28 ± 7	8 ± 2	7 ± 1
Lysolipids	11 ± 1	2 ± 1[b]	25 ± 9	10 ± 3[b]
Cholesterol/phospholipid	0.26	0.12	0.41	0.28

[a]Results represent mean values ± S.D. of four separate analyses of pooled samples, expressed as mg lipid per 100 ml of plasma or erythrocyte packed cell volume.
[b]Significantly lower ($P < 0.05$) than corresponding control value (Rank test).
[c]Significantly higher ($P < 0.05$) than corresponding control value.

TABLE 3

Relative Distribution of Fatty Acids Obtained from Phospholipids of Erythrocytes and Plasmas of Control and *P. vinckei*-Infected Mice[a]

Fatty acid	Fatty acid (wt % of total)[b]			
	Erythrocytes from control mice	Erythrocytes from infected mice	Plasma from control mice	Plasma from infected mice
14:0	1.8 ± 0.2	1.4 ± 0.2	2.2 ± 0.7	3.2 ± 1.5
14:1	0.3 ± 0.1	0.6 ± 0.4	0.2 ± 0.2	0.3 ± 0.1
16:0	36.0 ± 1.0	30.9 ± 1.6[d]	23.4 ± 3.1	19.7 ± 0.5
16:1	nd[c]	nd	nd	nd
18:0	8.3 ± 1.6	4.2 ± 0.3[d]	9.8 ± 1.3	7.3 ± 0.6
18:1	12.8 ± 2.1	10.3 ± 0.1	9.1 ± 0.7	11.1 ± 0.2
18:2	23.1 ± 1.8	31.9 ± 1.7[e]	38.6 ± 3.7	46.4 ± 1.4[e]
18:3	nd	nd	nd	—
20:2	nd	nd	nd	—
22:0	0.7 ± 0.1	2.3 ± 0.8	1.5 ± 0.4	0.6 ± 0.6
20:4	9.3 ± 2.9	15.0 ± 2.1	5.8 ± 3.9	7.5 ± 0.3
22:6	4.0 ± 1.0	2.8 ± 0.8	3.7 ± 0.8	3.6 ± 0.5

[a]Results represent mean values ± S.D. of four separate analyses.
[b]Fatty acids are designated by number of carbon atoms:number of double bonds.
[c]nd, Not detectable.
[d]Significantly lower ($P < 0.05$) than corresponding control value (Rank test).
[e]Significantly higher ($P < 0.05$) than corresponding control value.

on the other, to the RBC content of total peroxidizable lipid (16). The total amounts of vitamin E and malonyldialdehyde detected were about threefold and eightfold higher, respectively, in RBC from infected compared with control mice, when expressed on a cellular basis (Table 6). However, when expressed per unit of peroxidizable lipid, the amount of malonyldialdehyde detected in incubations of RBC from infected mice was barely higher than, and the vitamin E level was only half, that in control RBC (Table 6).

DISCUSSION

Changes in total plasma lipids in response to a malarial infection have not revealed a consistent trend and appear to depend on the experimental model used. In infections with *P. falciparum, P. knowlesi* and *P. lophurae*, for example, total lipids in plasma were reported to increase (3,30,31), while others (32) have reported a decrease in plasma from rats infected with *P. berghei.* Our results in mice infected with *P. vinckei* (Table 2) agree with the

TABLE 4

Relative Distribution of Fatty Acids Obtained from Neutral Lipids of Plasmas of Control and *P. vinckei*-Infected Mice[a]

Fatty acid	Fatty acid (wt % of total)	
	Plasma from control mice	Plasma from infected mice
14:0	2.1 ± 1.4	2.7 ± 0.3
14:1	0.2 ± 0.2	0.5 ± 0.3
16:0	13.3 ± 1.0	13.6 ± 0.6
16:1	4.4 ± 1.0	5.5 ± 0.8
18:0	1.6 ± 0.8	2.0 ± 0.8
18:1	18.0 ± 1.5	18.0 ± 1.5
18:2	49.6 ± 2.5	50.0 ± 5.2
18:3	4.5 ± 0.7	1.4 ± 1.4
20:2	0.2 ± 0.2	0.8 ± 1.2
22:0	0.4 ± 0.5	0.4 ± 0.5
20:4	5.6 ± 1.0	3.5 ± 0.8[b]
22:6	0.8 ± 0.4	0.8 ± 0.2

[a]Abbreviations as in Table 3. Values are mean ± S.D. from four separate analyses.

[b]Significantly lower ($P < 0.05$) than corresponding control value (Rank test).

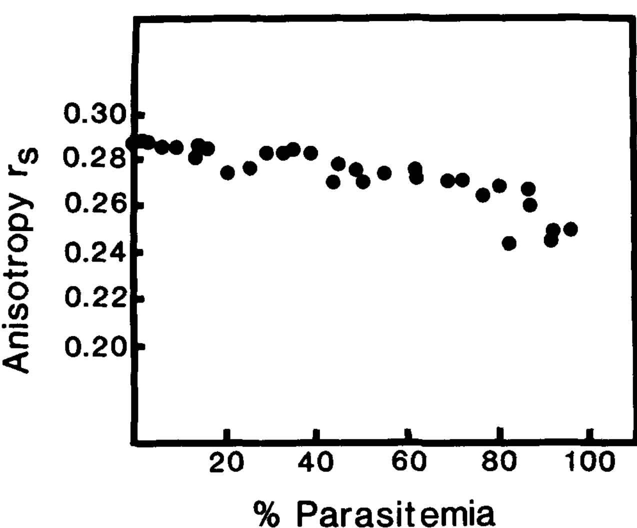

FIG. 1. Steady-state fluorescence anisotropy, r_s, of TMA-DPH as a function of parasitemia. Each point is the mean of triplicate experiments. The r_s value is corrected for light scattering effects and the response of the emission monochromator and photomultiplier tube system.

TABLE 5

Relative Degree of Saturation of Fatty Acids of Erythrocytes of Control and *P. vinckei*-Infected Mice[a]

Saturation	Erythrocytes from control mice	Erythrocytes from infected mice
Saturated	45.9 ± 0.4[b]	39.6 ± 2.1[c]
Monounsaturated	14.2 ± 0.5	10.7 ± 10.5[c]
Polyunsaturated	38.5 ± 1.9	49.7 ± 2.1[d]

[a]Percentages are calculated from results shown in Tables 3 and 4.

[b]Results are expressed as wt % of the total fatty acids (mean ± S.D.).

[c]Significantly lower ($P < 0.05$) than corresponding control value (Rank test).

[d]Significantly higher ($P < 0.05$) than corresponding control value.

TABLE 6

Ratio of the Vitamin E Content and Spontaneous Malonyldialdehyde Production in *P. vinckei*-Infected vs Control Erythrocytes in Relation to Various Parameters

Data expressed in relation to	PRBC/CRBC (ratio)[a]	
	MDA formed	Vitamin E
Cell number or packed cell volume	8.1[b]	2.8[c]
Total lipid	2.7[d]	0.9
Phospholipid	1.9	0.7
PUFA	1.4	0.5

[a]Abbreviations: PRBC, parasitized red blood cells; CRBC, control red blood cells; MDA, malonyldialdehyde; PUFA, polyunsaturated fatty acids.

[b]CRBC and PRBC form spontaneously 5.7 ± 1.4 and 46.2 ± 7.8 nmol MDA/10^{10} cells/hr, respectively.

[c]CRBC and PRBC contain 1.5 ± 0.5 and 3.8 ± 1.0 μg vitamin E/ml packed cells, respectively.

[d]Ratios are calculated from results shown in Table 1.

latter report. In general, the changes in individual components of the total lipids and fatty acids within plasma were small and paralleled the more profound changes observed in RBC (Tables 1, 3). The small decrease in total plasma lipids was due mainly to lower circulating levels of neutral lipids. Plasma triglycerides showed a small increase, which could be the result of increased levels of triacylglyceride-rich lipoproteins, such as chylomicrons and very low density lipoproteins, which are elevated in the serum of animals infected with various strains of *Plasmodium* (33) and may, in fact, precede the very large increase in triglycerides observed within RBC upon infection (Table 2). The hypertriglyceridemic state observed in murine malaria has been attributed to a parasite-induced product, released by reticuloendothelial cells, that inhibits lipoprotein lipase, resulting in suppressed triglyceride clearance (34). It is interesting to note that this factor, previously known as cachectin, has recently been suggested to be identical with tumor necrosis factor (35), a macrophage-derived mediator that has also been implicated in causing the intraerythrocytic death of malarial parasites (36).

The lipid analysis of RBC from control and *P. vinckei*-infected mice confirmed some aspects of previous reports of work in which other host species and strains of *Plasmodium* (2–6) were used, i.e., that the total amounts of lipids and phospholipids are increased in malaria-infected RBC (Table 1), although numerous differences in lipid composition were seen. It is difficult to assign changes in the composition of individual lipids to particular membranes, since the experimental material consisted of a multicompartment membrane system, containing the plasma membranes of the host and parasite, the membranes of the parasitophorous vacuole and the intracellular membranes of various plasmodial organelles. We did not attempt to analyze lipids in isolated parasites, since most published methods of isolation yield parasite fractions that either lack parasite membranes or are contaminated with RBC components (37). Other methods, such as those described by

Nillni et al. (38), were found to be inapplicable to *P. vinckei* (Stocker, R., unpublished observations). Despite these difficulties, there seems to be a consensus that "free parasites" contain more than 60% of the total lipid of the parasitized RBC (3,26,27). If this is the case, a major quantitative contribution to the changes we have observed in *P. vinckei*-infected RBC is likely to be the rapid proliferation of plasmodial membrane lipids as infection proceeds, although the observations with TMA-DPH also imply qualitative changes in the RBC plasma membrane. Notable were the several-fold increases in the levels of triglyceride and PC, a finding consistent with enhanced metabolism of glucose by the parasite to form the intermediate α-glycerol-3-phosphate that acts as a common precursor for de novo biosynthesis of glycerides and phospholipids (39). Less than 5% of the glucose metabolized by the parasite is used for de novo synthesis of fatty acids and sterols like cholesterol (39). This small contribution, taken in conjunction with decreased plasma levels of cholesterol, may explain why the relative concentration of cholesterol decreased from 15 to 10% (Table 1) and why the cholesterol-to-phospholipid ratio decreased in RBC from infected mice (Table 2).

As mentioned above, the parasite exhibits at least a partial dependence upon the extra-erythrocytic environment for its supply of fatty acids. Therefore, the changes observed in the relative concentrations of the fatty acids in phospholipids of infected vs control RBC (Table 3) may be a reflection of the uptake of these compounds from the plasma. This view agrees with the finding that changes in the fatty acid composition of RBC phosphoglycerides from infected animals parallel those observed in the plasma (Table 3). It is important to note that *P. vinckei* infection is associated with an increase in the relative amounts of polyunsaturated fatty acids at the expense of the saturated palmitic and stearic acids in RBC (Tables 3 and 5). Although this does not agree with findings in *P. lophurae* infection in ducks (3), where palmitic and stearic acids were elevated in parasitized RBC, it does parallel the decreases in stearic acid content observed in mouse and monkey RBC infected with *P. berghei* and *P. knowlesi*, respectively (5,6). Furthermore, there are differences in the distribution patterns of the unsaturated fatty acids within parasitized RBC from different combinations of host and plasmodial strains. For example, infections with *P. lophurae* and *P. knowlesi* result in increases in the monounsaturated oleic acid (3,6), while the relative abundance of the polyunsaturated linoleic and arachidonic acids increases within *P. vinckei*-infected RBC (Table 3).

The increases in the total and relative amounts of polyunsaturated fatty acids within parasitized RBC can largely explain why, on a cellular basis, they produce eight times more malonyldialdehyde than do their control counterparts: when analyzed in relation to the increased availability of peroxidizable lipid, there is only a small difference between the two cellular systems in their amounts of lipid autoxidation (Table 6). The latter argument is supported by our recent finding that in neither control nor *P. vinckei*-infected RBC is there any net accumulation of α-tocopherolquinone, one of the oxidation products of vitamin E (40).

Under the experimental conditions used in this study, the observed decrease in steady-state fluorescence anisotropy (r_s) of TMA-DPH in infected RBC (Fig. 1) is probably attributable solely to alterations in the outer leaflet of the RBC plasma membrane (28,29). This change in r_s is interpreted as representing a decreased "order" in the lipids of that compartment (41,42). In other systems, oxidation of membrane lipids results in the depletion of polyunsaturated fatty acids, with concomitant increases in the relative concentrations of saturated (43–45) and shorter chain (45) fatty acids, and an increased "order" of the membrane lipids (43–46). Furthermore, while treatment of RBC with t-butyl hydroperoxide increases their negative surface charge (46), infection of RBC with *P. vinckei* results in a more positive surface charge (47). Taken together, these contrasts cast doubt upon the occurrence of reactive oxygen-induced damage to lipid in *P. vinckei*-infected erythrocytes during the natural course of the infection. "Protection" of the polyunsaturated fatty acids from oxidation may be a consequence of compensatory changes in the antioxidant systems of the parasitized RBC (15,40,48).

As discussed above, most of the observed increase in the unsaturated fatty acid content of infected RBC can be attributed to the presence of parasite membranes. This may explain why they are more susceptible to peroxidative damage than their more saturated counterparts in the host red cell membrane and why the spontaneous malonyldialdehyde formation increases linearly with increasing parasitic load in parasitized RBC (15). Also relevant to the question of increased susceptibility of the parasite lipids to peroxidation is the observation that the relative concentration of the main lipid-soluble antioxidant, vitamin E, is actually decreased in parasitized RBC when it is expressed per unit of polyunsaturated fatty acid (Table 6). With respect to membrane lipids associated with the parasite, such a decrease in the relative amount of vitamin E may well be underestimated, since it has been suggested that the vitamin E detected in *P. vinckei*-infected RBC is associated mainly with the host cell membrane (15).

Thus, we conclude that the relative increase in unsaturated fatty acids and the relative decrease in vitamin E may act synergistically to increase parasite membrane susceptibility to peroxidative damage vis-a-vis that of the host red cell membrane. The possible relevance of this observation to the occurrence of "crisis forms," i.e., degenerating malaria parasites observed within intact RBC (49), remains to be established.

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METHODS

A Micromethod for the Estimation of Blood Dolichol

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A micromethod for the estimation of dolichol in blood was developed using high performance liquid chromatography. This method can be applied to whole blood or plasma. With detector sensitivity set at 0.005 Absorbance Unit Full Scale, samples as little as 50 μl are sufficient to carry out the determination. Blood samples from the tail vein of inbred strains of C57 BL/6 NNia-1 mice 3, 6, 12 and 24 mo of age were examined. Blood dolichol levels decreased with age from 163.3 ng/ml at 6 mo to 110.1 ng/ml at 24 mo. The major dolichol homologs were C-85 (10.4%), C-90 (41.6%), C-95 (38.0%) and C-100 (8.1%).
Lipids 22, **58–60 (1987).**

Dolichols are very long chain 2,3-dihydropolyprenols that occur ubiquitously in eucaryotes. In biological fluids and tissues they exist in various forms including phosphate esters, free alcohols and esters of fatty acids (1). In the form of dolichyl phosphate, these compounds function as saccharide carriers for biosynthesis of asparagine-linked glycoprotein (2).

Recently, there have been two reports on the determination of dolichol in blood (3,4). One study by Elmberger and Engfeldt (3) showed an age-associated increase in plasma dolichol, while the other by Yamada et al. (4) showed a decrease. Work from this and other laboratories (5–9) has shown an age-associated increase in the level of unesterified dolichol in various tissues. With focus on a biomarker for aging, we set out to investigate the age-associated changes in dolichol levels in the blood of an inbred strain of mice.

Currently, estimation of plasma dolichol requires samples of 1 to 10 ml (3,4) and the use of a fluorescent derivative to improve sensitivity (4). Since we were interested in working with blood samples from mice, it became necessary to develop a method that can use small volumes of blood from the tail vein. In this paper, we report a very sensitive method that is simple, fast and does not use derivitization, for the determination of blood dolichol.

MATERIALS AND METHODS

Chemicals and reagents. Dolichol standards were obtained from Sigma Chemical Co. (St. Louis, Missouri). Dolichol C-125 (internal standard) was purchased from the Polish Academy of Science (Warsaw, Poland). Reversed phase cartridge columns (SPICE) were obtained from Analtech (Newark, Delaware). All solvents were obtained from J.T. Baker Co. (Bethlehem, Pennsylvania) and were either HPLC grade or resi-analyzed. The high performance liquid chromatography (HPLC) column was from Rainin Instrument Co. (Woburn, Massachusetts).

Animals. C57 BL/6 NNia-1 mice aged 3, 6, 12 and 24 mo were obtained from Charles River Laboratories (Wilmington, Massachusetts) and were fed a commercial diet and water ad libitum.

Quantification of dolichol. Blood samples (50–200 μl) from the tail vein of the mice were collected into heparinized calibrated micropipets and transferred to 18-ml screw-cap culture tubes. One ml 2 N NaOH in methanol and 50 ng internal standard, dolichol C-125, were added. The tubes were capped, and their content was mixed thoroughly and heated at 70–75 C for 1 hr with intermittent mixing. The mixture was cooled and neutralized with 1 N HCl, and the unsaponifiables were extracted twice with 5 ml chloroform. The pooled extract was evaporated at 30 C under nitrogen. The dolichol was taken up in 0.5 ml $CHCl_3$/MeOH (2:1, v/v) and applied in 1–2 ml methanol on a C18 reversed-phase cartridge (SPICE) prewashed with 10 ml MeOH/H_2O (75:25, v/v), 10 ml acetone and 10 ml methanol in that order. The sample was washed with 15 ml methanol and the dolichol eluted with 10 ml acetone. The acetone fraction was taken to dryness and redissolved in 50 μl mobile phase, methanol/isopropanol/hexane (44:44:12, v/v/v). Ten μl of this preparation was used for HPLC analysis.

HPLC system. The HPLC system consisted of a Spectra-Physics 8700 solvent delivery system, a Rheodyne model 7125 sample injector, a Beckman model 160 fixed wavelength UV detector (214 nm, Zinc Lamp), a Rainin reversed-phase C18 column (Microsorb 3 μm, 0.46 × 10 cm) and a Spectra-Physics 4100 computing integrator. All runs were done isocratically using a mobile phase consisting of methanol/isopropanol/hexane (44:44:12, v/v/v) at 0.5 ml/min. All solvents were degassed continuously with helium. The detector sensitivity was set at 0.005 Absorbance Unit Full Scale (AUFS). The analytical procedure was linear at least from 0.5–400 ng per 10 μl of sample injected.

RESULTS AND DISCUSSION

Preliminary experiments in which alkaline concentration, temperature and time of saponification of blood samples were varied showed that heating samples at 70–75 C in 2 N methanolic NaOH for 1 hr was optimum for getting rid of interfering lipids, such as cholesteryl esters, and for dolichol recovery. The recovery of the internal standard after saponification was >95%. Furthermore, results from samples prepared in the absence or presence of pyrogallol (2.5 mg/ml) showed no significant differences; hence the experiments described were done in the absence of pyrogallol.

Figure 1 shows HPLC of dolichols obtained from a 100-μl blood sample, of which an equivalent of 20 μl was injected using detector sensitivity set at 0.005 AUFS. All the major homologs are well separated from each other and from the internal standard. The predominant dolichol homologs are C-85 (10.4%), C-90 (41.6%), C-95 (38.0%) and C-100 (8.1%). All other homologs combined represent less than 3% of the total.

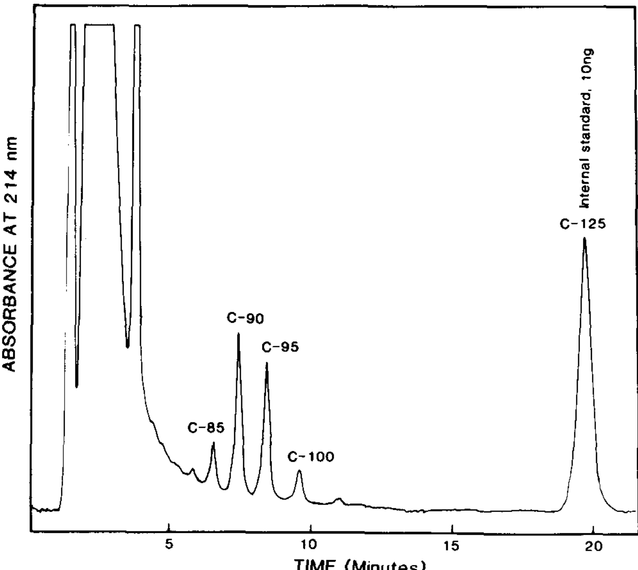

FIG. 1. High performance liquid chromatographic analysis of total dolichol from a 100-µl blood sample of which an equivalent of 20 µl was injected. The internal standard represents 10 ng of dolichol C-125. Conditions of analysis are described in Materials and Methods. The number on peaks indicates the total number of carbons in each dolichol homolog.

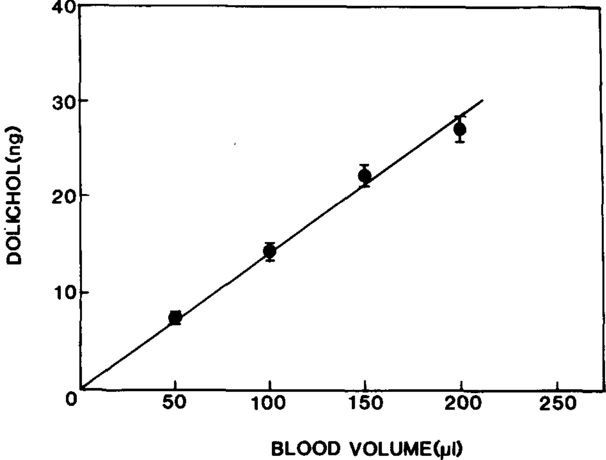

FIG. 2. Calibration curve for blood dolichol of C57 BL/6 NNia-1 mice using the method described in Materials and Methods. Each point is the mean ± S.E. of three replicates.

TABLE 1

Effects of Age on Total Dolichol (ng/ml ± SD) of Blood from C57 BL/6 NNia-1 Mice

Age (mo)	Dolichol (ng/ml)
3(4)[a]	164.2 ± 17.7
6(4)	163.3 ± 22.8
12(4)	143.3 ± 12.5
24(4)	110.1 ± 17.8

[a]Number of animals given in parentheses.

Figure 2 shows a calibration curve obtained by analyzing different amounts of blood (50–200 µl) from 12 mo-old C57 BL/6 NNia-1 mice. The standard deviation for triplicate determinations of the same sample from 50–200 µl blood samples was between 8 and 11% with standard error of 7% or less of the values. The linearity of this graph shows that the procedure is good for at least a 200 µl blood sample.

Table 1 shows results obtained by analyzing blood from 3, 6, 12 and 24 mo-old C57 BL/6 NNia-1 mice. There is a decrease in blood dolichol levels from 163 ng/ml at 6 mo to 110 ng/ml at 24 mo. This age-associated decrease agrees with results obtained by Yamada et al. (4) for human plasma. A possible explanation for this decrease may be a slowdown of de novo biosynthetic processes with age (10).

In addition, we have observed that plasma contributes about 50% and 62% dolichol to the blood dolichol pool of C57 BL/6 NNia-1 mice and human, respectively and that approximately one-third of the dolichol in the blood of C57 BL/6 NNia-1 mice is unesterified.

The method described here is reliable and can be easily adapted for plasma or serum dolichol levels. Compared to other methods (3,4), this procedure is less time consuming and more sensitive. Blood dolichols can be estimated from samples as little as 50 µl compared to samples of at least 1 ml and formation of derivatives for improved sensitivity is not required. The increased sensitivity of the described method is due to the use of highly end-capped 3 µm columns, a fixed wavelength detector of 214 nm that gives a stable baseline at 0.005 AUFS and continuous degassing of the solvents with helium to reduce drift in base line. Sensitivity may also be improved by employing a normal phase HPLC column (11), but a major drawback of this technique is its inability to resolve dolichol homologs.

Useful applications of this method may be found in certain conditions, metabolic or otherwise, in which it may be necessary to monitor the dolichol levels without killing the animal in question or in cases where sample size and availability are minimal, for example, with newborns or elderly subjects. For certain neurological disorders, such as ceroidlipofuscinosis (12), alcoholic syndrome in infants born to alcoholic mothers (13) and metastatic carcinoma (14), dolichol levels of the urine provide valuable information. Blood is more readily available and more stable in metabolite concentrations than urine. By our method, it may be possible to monitor these and other disorders through the levels of dolichol in the blood.

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Improved Enzymatic Hydrolysis of Conjugated Bile Acids

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The hydrolysis of conjugated bile acids by cholylglycine hydrolase (EC 3.5.1.24) using the standard procedure for a commercial enzyme preparation was found to be incomplete, as judged by the use of 24-^{14}C-taurocholic acid as a tracer. A method is proposed that incorporates the nonionic detergent Triton X-100 into the reaction mixture to achieve almost complete hydrolysis. It is proposed that the observed enhancement of enzyme activity is due to the formation of micelles by the detergent.
Lipids 22, **61–63 (1987).**

Prior to the introduction of an enzymatic hydrolysis procedure by Nair and Garcia (1), the method of choice for hydrolysis of bile acid conjugates was 1 N NaOH at elevated temperature and pressure (2). Cholylglycine hydrolase (EC 3.5.1.24) offers a much milder alternative to hot alkali.

When an enzymatic reaction is to be applied to prepare a substrate for further manipulations—for example, derivitization and gas chromatographic analysis—two criteria may be used to judge the suitability of the enzyme for the task at hand. The first is the speed or rate of the reaction; this is primarily a function of the enzyme concentration, pH and temperature. The second is the completeness of the reaction; this is a function of the thermodynamic equilibrium between substrate and product and is independent of the enzyme.

In the course of our investigations on the utility of serum bile acid analysis in the diagnosis of hepatobiliary disease, we observed that when ^{14}C-labeled taurocholic acid was used as a tracer, the enzymatic hydrolysis of taurocholic to cholic acid was not complete. The addition of the nonionic detergent Triton X-100 to the reaction mixture resulted in complete hydrolysis. In cooperation with Davis and coworkers, we have used this method in the determination of bile acids in hepatocyte cell culture fluid (3).

MATERIALS AND METHODS

24-^{14}C-Taurocholic acid (52.0 mCi/mmol, New England Nuclear, Boston, Massachusetts) was judged to have a radiochemical purity of greater than 95% by thin layer chromatography (TLC) (silica gel–impregnated glass fiber paper [Gelman Sciences, Ann Arbor, Michigan], solvent system of isobutanol/acetic acid/water, 80:1:1, v/v/v) and was used without further purification. Conjugated bile acids (synthetic) were from Sigma Chemical Co. (St. Louis, Missouri) and were judged homogeneous using the TLC system above. Triton X-100 (scintillation grade) from Eastman Kodak Co. (Rochester, New York) was purified by dissolving 10 ml Triton in 100 ml 0.1 N KOH and extracting with two 100-ml portions of ethyl acetate and removing the solvent on a rotary evaporator. Ethyl acetate (analytical reagent) from Mallinckrodt Chemical Works (St. Louis, Missouri) was redistilled before use. Cholylglycine hydrolase (partially purified, 107 units/mg protein) was from Sigma. A stock solution of enzyme was prepared by dissolving 7,500 units of enzyme in 7.5 ml of distilled water. Aliquots of 0.05 ml were dispensed into plastic tubes, tightly sealed and stored at −20 C. The working solution of enzyme was prepared immediately before use by adding 1.0 ml 0.025 M acetate buffer, pH 5.6, to a tube of stock solution. All other materials were reagent grade and were used without further purification.

Ten ml of Pico-fluor-30 liquid scintillation cocktail from Packard Instrument Co. (Downers Grove, Illinois) was added to each sample and then counted for 10,000 counts on a Packard Model 3380 liquid scintillation counter, using an external standard radiation source for quench corrections.

RESULTS

Ca. 6000 dpm of radiolabeled taurocholic acid was added to a protein-free extract of 0.1 ml of normal human serum prepared by adding 1.0 ml ice-cold ethanol/methanol/dimethoxypropane (2:2:1, v/v/v), centrifuging to remove the precipitated protein, decanting and drying the supernatant under a stream of dry nitrogen. Hydrolysis was carried out according to instructions from Sigma Chemical Co. (Table 1). Cholylglycine hydrolase was used at a nominal concentration of 47.6 U/ml. The incubation temperature was 37 C. At the end of the incubation, each sample was acidified by the addition of 0.1 ml 2 N HCl and extracted with 2 × 2 ml ethyl acetate. Under these conditions, cholic acid is extracted into the organic phase, while taurocholic acid remains in the aqueous phase. The ^{14}C content of each fraction was measured and the percentage of 24-^{14}C-taurocholic acid hydrolyzed was determined. The results are shown in Table 2. The experiment was then repeated with the addition of 0.2 ml 0.25% (v/v) Triton X-100 in distilled water. The results are shown in Table 3.

To determine the amount of conjugated bile acid that could be completely hydrolyzed under these conditions, the experiment was repeated after the addition of varying amounts of unlabeled taurocholic or taurochenodeoxycholic acids, ranging from 0.01 to 1.0 μmol/tube. The results are shown in Figure 1.

TABLE 1

Reagents for the Enzymatic Hydrolysis of Conjugated Bile Acids

Reagent	Ml per tube
Acetate buffer (0.025 M, pH 5.6)	0.2
0.78% 2-Mercaptoethanol	0.2
1.86% EDTA	0.2
Cholylglycine hydrolase (47.6 U/ml)	0.2

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TABLE 2

Time Course for the Hydrolysis of 24-^{14}C-Taurocholic Acid

	1 hr	5 hr	16 hr
Hydrolyzed (%)	83.0[a]	84.4[a]	87.4
Unhydrolyzed (%)	17.0	15.6	12.6

[a]Each data point is the mean of duplicate determinations.

TABLE 3

Time Course for the Hydrolysis of 24-^{14}C-Taurocholic Acid in the Presence of Triton X-100[a]

	0 min	15 min	30 min	45 min	60 min
Hydrolyzed (%)	0.2[b]	97.9	98.1	98.1	98.5
Unhydrolyzed (%)	99.8	2.1	1.9	1.9	1.5

[a]0.2 ml 0.25% Triton X-100 was added to each hydrolysis mixture.
[b]Each data point is the mean of duplicate determinations.

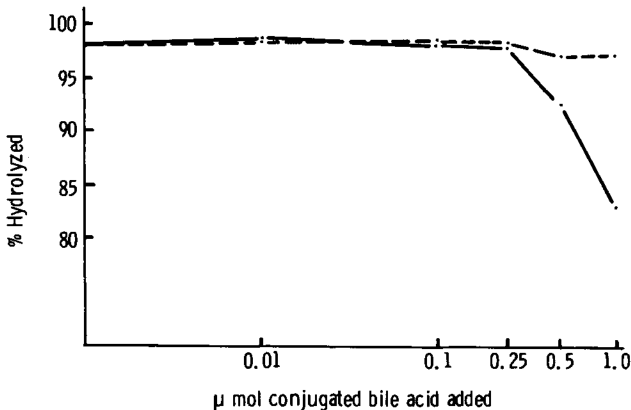

FIG. 1. Effect of excess taurine-conjugated bile acids on the hydrolysis of 24-^{14}C-taurocholic acid. ———, Percentage hydrolyzed after the addition of unlabeled taurochenodeoxycholic acid; ______, Percentage hydrolyzed after the addition of unlabeled taurocholic acid.

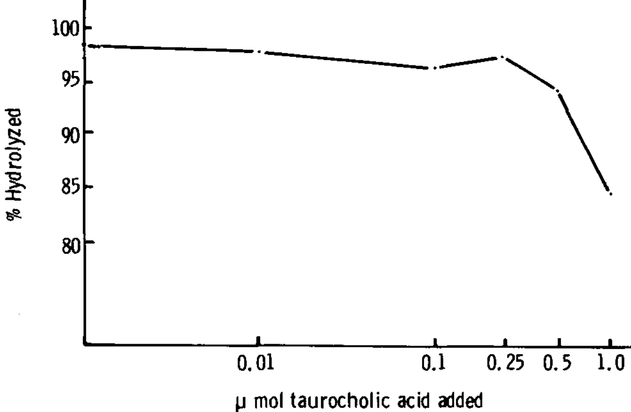

FIG. 2. Effect of excess taurocholic acid on the hydrolysis of 24-^{14}C-taurocholic acid without precipitation of serum proteins.

To test for any inhibitory effects of serum proteins, the experiment was repeated using 0.1-ml aliquots of untreated human serum spiked with 0.01 to 1.0 μmol/tube unlabeled taurocholic acid. The results are shown in Figure 2.

To confirm the nature of the extractable radioactivity, triplicate samples of 24-^{14}C-taurocholic acid were subjected to enzymatic hydrolysis in the presence of Triton X-100 and were extracted with ethyl acetate under acidic conditions as described. The ethyl acetate was removed under a stream of nitrogen. The residue was dissolved in a minimal volume of methanol and subjected to TLC (silica-impregnated glass fiber paper, solvent system of isooctane/isopropyl alcohol/isopropyl ether/acetic acid, 20:1:1:1, v/v/v/v). More than 95% of the applied radioactivity co-migrated with authentic cholic acid (data not shown).

DISCUSSION

Cholylglycine hydrolase has been used extensively in the analysis of bile acids in biological materials (4–6). There remain, however, certain problems with the use of this enzyme as a reagent for the preparation of samples for subsequent manipulation, e.g., gas chromatography. Roovers et al. reported that, at low substrate concentrations (less than 60 nmol/ml), the hydrolysis rate slowed rapidly, with the effect being more pronounced with taurine than glycine conjugates (7). Studies in which the completeness of hydrolysis was assessed by the recovery of free bile acids from added conjugated bile acids have used higher concentrations of substrate (8–10). Furthermore, it is doubtful that the procedures used to assess analytical recovery (colorimetric determination of liberated amino acid or gas chromatographic determination of liberated bile acid using flame ionization detection) possess adequate sensitivity to determine if a small amount of conjugated bile acid was left unhydrolyzed.

The literature reflects a substantial variation in the amount of enzyme used to prepare biological samples for chromatographic analysis of their bile acid content. The amount of cholylglycine hydrolase used is not stated in some reports (1,5); in others it is reported simply as being "in excess" (8). Where the amount of enzyme is stated, the amounts range from <10 units per tube (6,7,10,12) to >200 units per tube (4,12). In light of these discrepancies, we originally thought that complete hydrolysis could be achieved using higher concentrations of enzyme. However, our attempts failed even when the concentration of enzyme was fivefold that shown in Table 1 and the incubation time was extended to 24 hr (data not shown). These results are in accordance with theory, as the amount of enzyme present should not affect the equilibrium between substrate and product, but merely the speed at which equilibrium is established.

Our goal was to develop a gas chromatographic analysis using an electron capture detector, which could be performed on the limited volumes of serum (0.1 ml or less) obtainable from pediatric or geriatric patients. Thus, the behavior of the enzyme relative to small amounts of substrate was critical. We therefore used radiolabeled substrate, by which very small amounts of materials could be monitored easily.

Table 2 shows that, even after a 16-hr incubation, an unacceptable unhydrolyzed fraction of bile acid remained. Table 3 shows that after the addition of 0.25% (v/v) Triton X-100, the reaction proceeded essentially to completion in 1 hr. Under these conditions (9.5 units cholylglycine hydrolase per tube, incubation at 37 C for 1 hr and 0.2 ml 0.25% [v/v] Triton X-100 per tube), up to 0.25 μmol/tube of conjugated bile acid was hydrolyzed without interference from serum proteins (Figs. 1 and 2).

The increase in extractable radioactivity is due to an increased liberation of cholic acid and not merely to a change in the partition coefficient of taurocholic acid in the water/ethyl acetate system. This is demonstrated in two ways: first, there is no increase in extractable radioactivity, due to labeled taurocholic acid in the presence of Triton X-100 (zero time point, Table 3); and second, the extracted radioactivity co-migrates with cholic acid on TLC.

The mechanism for enhancement of the hydrolysis of bile acid conjugates by cholylglycine hydrolase in the presence of Triton X-100 is unknown. However, since the concentration of Triton X-100 in the reaction is in excess of its critical micellular concentration (11), it may enhance the incorporation of bile acid conjugates into the micelle, with the polar conjugate portion exposed for enzyme action. The equilibrium between conjugated and free bile acids is then altered by the sequestration of the free bile in the micelle.

We conclude that when cholylglycine hydrolase is to be used as a reagent for the preparation of free bile acids for further manipulation, Triton X-100 should be included in the reaction mixture to ensure hydrolysis near 98%.

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Tissue Distribution of Phytanic Acid and Its Analogues in a Kinship with Refsum's Disease

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Three of six kin were identified, by high performance thin layer chromatography, capillary gas chromatography and mass spectrometry, as having phytanic acid storage disease. Phytanic acid was found in triacylglycerol and, to a lesser degree, in phosphatidylcholine and free fatty acids. An unsaturated analogue of phytanic acid was additionally identified in plasma and erythrocyte triacylglycerols. In plasma, branched chain fatty acids were primarily localized in the low density lipoprotein fraction. The concentration of plasma major fatty acids was not affected by the presence of these branched chain fatty acids. In contrast to plasma, only small amounts of phytanic acid were found in cerebrospinal fluid and biopsied sural nerve. The nerve phytanate was mainly associated with triacylglycerol in epineurial and perineurial tissues. Lack of phytanate accumulation in sural endoneurium, even in cases with severe fiber degeneration, suggests that demyelination in Refsum's disease may not be due to myelin instability resulting from the incorporation of branched chain fatty acids into peripheral nerve membrane.
Lipids 22, 69-75 (1987).

Refsum's disease is a rare inherited disorder of fatty acid oxidation characterized clinically by peripheral neuropathy, visual loss due to retinitis pigmentosa, icthyosis and bony abnormalities (1). The major pathway for phytanic acid oxidation in humans involves first, an unusual initial α-oxidation to yield α-hydroxy phytanic acid and then the n-1 fatty acid, pristanic acid; and second, a series of successive β-oxidation steps for the further degradation of pristanic acid (2-5). The level of plasma phytanic acid is markedly increased in patients with Refsum's disease (6). Normally, it is virtually undetectable (7). The metabolic error involved in Refsum's disease lies in failure of the converson of phytanic acid to α-hydroxyphytanic acid. This leads to widespread tissue storage of phytanic acid (8-10). The pathogenesis of nerve fiber degeneration in phytanic acid storage disease is unclear. It has been hypothesized that myelin stability may be affected by incorporation of branched chain fatty acids into tissue lipids in place of normal straight chain fatty acids (11,12). If this hypothesis of demyelination is correct, then endoneurial storage of phytanic acid might be expected. We have, therefore, carried out extensive biochemical studies on the plasma, erythrocytes, cerebrospinal fluid and biopsied sural nerve of patients from a kinship with Refsum's disease.

MATERIALS AND METHODS

Sample preparations. Heparinized blood samples (40 ml) were drawn from six kin (III-2, 70 years; III-4, 67 years; III-6, 66 years, III-8, 64 years; III-10, 62 years; and III-16, 59 years; see Fig. 1) and five age-matched normal subjects. After centrifugation, plasma was removed, and the packed cells were washed twice with isotonic saline solution. The method used to fractionate plasma into chylomicrons and fractions of very low density lipoprotein (VLDL, <1.006 g/ml), low density lipoprotein (LDL, 1.006-1.063 g/ml), high density lipoprotein (HDL,

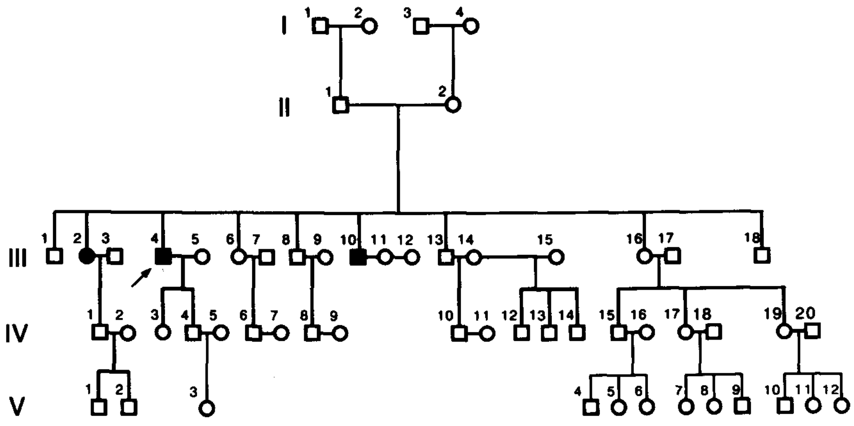

FIG. 1. Kinship of the three patients (III-2, III-4 and III-10) with Refsum's disease. Their clinically unaffected brother (III-8) and sisters (III-6 and III-16) do not show evidence of phytanic acid storage.

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1.063–1.210 g/ml) and very high density lipoprotein (VHDL, >1.210 g/ml) was essentially the same as that described by Hatch and Lees (13). A cerebrospinal fluid (CSF) sample was obtained from patient III-10, two normal subjects and 14 patients with various neurological disorders. All samples were stored at −70 C.

After obtaining informed consent, using a protocol approved by our Institutional Review Committee, a biopsy of sural nerve was performed, under local anesthesia, on four patients (III-2, III-4, III-6 and III-10) and four healthy volunteers. Immediately after removal, the sural nerve was desheathed on a cold plate (surface temperature, 10 C) under a dissecting microscope. The desheathed portion (endoneurium) contains myelin, Schwann cells, axons, capillaries and interstitial collagen, whereas the sheath (epi- and perineurium) consists mainly of perineurial cells, collagen, fibroblasts, elastic fibers, capillaries and lipocytes (14). The weights of both portions were recorded before and after lyophilization. The lyophilized samples were then stored at −70 C.

Clinical and neuropathologic observations. (As these findings will be presented in detail elsewhere, only the major clinical and neuropathologic observations are provided here.) Patient III-2 had retinitis pigmentosa, progressive restriction of visual fields, bony abnormalities (shortness of metatarsal bones), hearing loss and mild peripheral neuropathy (no obvious weakness or sensory loss but decreased knee and absent ankle reflexes). The number of myelinated fibers (MF) per mm^2 was 4327, which is below normal.

Patient III-4 had retinitis pigmentosa, peripheral visual field loss, short metatarsal bones and clinical (nerve conduction and electromyography) evidence of peripheral neuropathy. The number of MF/mm^2 was 3138—unequivocally below normal.

Patient III-10 had retinitis pigmentosa, restricted visual fields, progressive hearing loss, short metatarsal bones and symptoms and findings indicative of mild neuropathy. The number of MF/mm^2 was 4169, which is unequivocally abnormal. The size distribution of MF was bimodal (normal).

Patient III-6 is an unaffected sibling of patients III-2, III-4 and III-10. The number (6840/mm^2) and size distribution of MF of sural nerve were normal.

Lipid extraction and separation. Extraction of lipids from total plasma (1.0 ml), individual plasma lipoprotein fractions (equivalent to 2.0 ml plasma), erythrocytes (2.0 ml packed cells) and CSF was performed according to the procedure described by Nelson (15). The lipid extraction procedure used for biopsied sural nerve (~10 mg wet weight) was described by Michell et al. (16) and modified by us (17,18).

Separation of triacylglycerol into subclasses containing straight (TG_0) and branched chain (TG_1, TG_2) fatty acyl moieties was achieved by high performance thin layer chromatography (HPTLC) (Whatman, Clifton, New Jersey), using hexane/diethyl ether/acetic acid (90:12:1.5, v/v/v) as the developing solvent system. Total triacylglycerols ($TG_0 + TG_1 + TG_2$), cholesteryl ester and free fatty acids (FFA) were separated from total lipid extracts by TLC on Silica Gel G plates, using a developing solvent system of hexane/diethyl ether/acetic acid (75:23:2, v/v/v) (19). Separation of phospholipids into subclasses was achieved by TLC, using the solvent system described by Vitiello and Zanetta (20) and modified by us (21).

Neutral glycosphingolipids (NGL) were separated from erythrocyte lipids by column chromatography, using Sep-Pak silica cartridges (21). Before sample application, each cartridge was conditioned three times with 8 ml of $CHCl_3$. Lipid extracts (in 0.1 ml $CHCl_3$) from 1 ml of packed erythrocytes were applied to each cartridge. All nonpolar lipids were removed by elution with 40 ml of $CHCl_3$. The NGL were recovered from each cartridge by elution with 160 ml of acetone/methanol (9:1, v/v) mixture. Separation of NGL into monohexosylceramide (GL-1), lactosylceramide (GL-2), trihexosylceramide (GL-3) and globoside (GL-4) fractions was achieved by HPTLC, using the developing solvent system described by Vance and Sweeley (22).

Gas chromatography (GC) and mass spectrometry (MS). Before GC analysis, triacylglycerols and cholesteryl esters were transesterified using sodium methoxide reagent (23), while free fatty acids and phosphatidylcholine were methylated using BF_3-CH_3OH reagent as described by Morrison and Smith (24). Transesterification of total lipids was carried out using both sodium methoxide and BF_3-CH_3OH reagents. The resulting fatty acid methyl esters were further purified by TLC, using the developing solvent system described by Metcalf et al. (25).

The methylated samples were first analyzed on a Packard Gas Chromatograph, Model 419, equipped with dual hydrogen detectors. The operating conditions for GC were as described previously (19). The fatty acid methyl esters were also analyzed on a Hewlett-Packard capillary gas chromatograph, Model 5890A, equipped with a hydrogen flame ionization detector. A 30-m fused silica SP-2330 column, with 0.25 mm ID (Supelco, Bellefonte, Pennsylvania), was used. Each sample was run under an on-column injection mode from 100–240 C at a rate of 7 C/min with hydrogen as the carrier gas. Peaks on the chromatograms were identified by comparing the retention times with those of standard mixtures (Supelco) and were quantified by a Hewlett-Packard 3392 computing integrator using pentadecanoic acid (15:0, 0.5 mg/ml plasma) as an internal standard.

Phytanic acid and triunsaturated phytanic acid methyl esters were further identified by a Carlo Erba (Milan, Italy) gas chromatograph, Model 4160, directly interfaced via a glass capillary to a Kratos (Manchester, England) MS 50 double-focusing mass spectrometer. A 30-m fused silica DB-1 column, with 0.33 mm ID (J & W Scientific, Folsom, California), was used. Samples were applied via on-column injection and the GC was programmed from 140–250 C at 5 C/min. MS ionization was induced by electron bombardment at 70 eV with the source temperature at 200 C. The MS was scanned at 3 sec/decade, from m/z 500-30, at a resolution of 10,000. Data was acquired and analyzed by a Kratos DS55 system.

Plasma lecithin:cholesterol acyltransferase (LCAT). The method used to assay the in vitro rate of cholesterol esterification (LCAT activity) in plasma has been described by us (26). The LCAT activity was expressed either as the percentage of labeled cholesterol esterified per hour or as nmoles of cholesteryl ester formed per ml of plasma per hour. The radiolabeled cholesteryl esters formed during LCAT reaction were isolated by TLC, as described above. The method used to measure the distribution of radioactivity among the different fatty acid fractions of cholesteryl esters was that of Morris (27), as modified by Goodman and Shiratori (28).

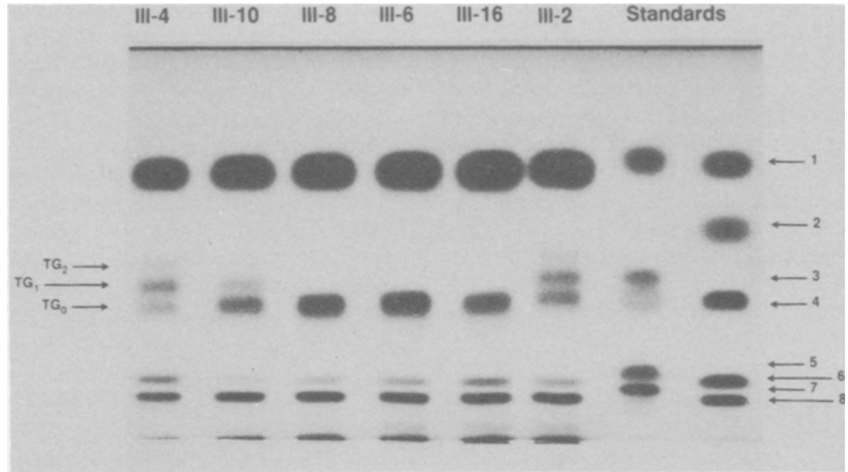

FIG. 2. HPTLC of plasma nonpolar lipids. TG_0, Normal triacylglycerol; TG_1, triacylglycerol with one branched chain fatty acid; TG_2, triacylglycerol with two branched chain fatty acids; 1, cholesteryl ester; 2, methyl palmitate; 3, 1,2-dipalmitoyl-3-phytanyl glycerol; 4, triacylglycerol; 5, phytanic acid; 6, palmitic acid; 7, phytol; 8, cholesterol. Lipid standard mixture (1 to 8) was purchased from Analabs/The Foxboro Co. (North Haven, Connecticut).

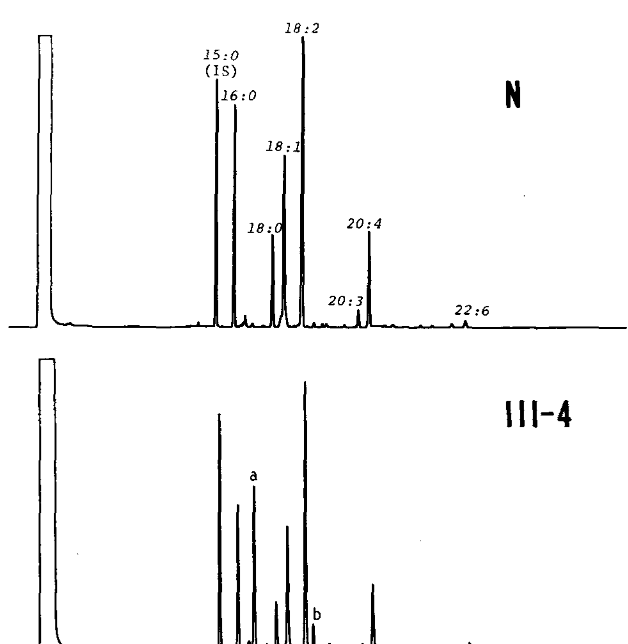

FIG. 3. Capillary gas chromatograms of fatty acid composition of plasma total lipids. N, Normal subject; III-4, affected patient; a, phytanic acid; b, triunsaturated phytanic acid.

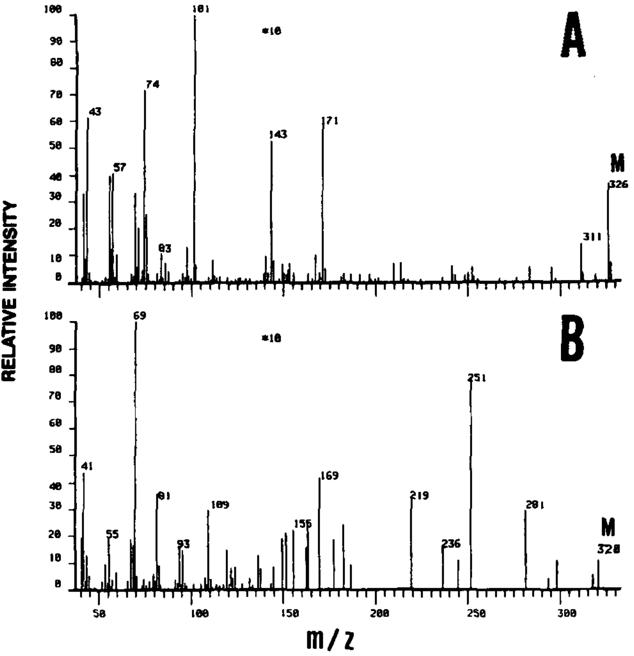

FIG. 4. Mass spectra of the branched chain fatty acid methyl esters from plasma lipids of patient III-4. A and B represent mass spectra of peaks a (phytanate) and b (triunsaturated phytanate) from Fig. 3, respectively.

RESULTS

Plasma lipids. By thin layer separation, two additional bands, which moved just above normal triacylglycerol (TG_0), were demonstrated in three of the six siblings (patients III-2, III-4 and III-10) (Fig. 2). These two fast-moving bands (TG_1 and TG_2) were triacylglycerols containing one and two molecules of branched chain fatty acids, respectively. By capillary GC (Fig. 3) and MS (Fig. 4) analyses, two types of branched chain fatty acids, phytanic acids and triunsaturated phytanic acid, were demonstrated and identified by their characteristic base peaks (Fig. 4) (m/z 101 and m/z 69, respectively) and molecular ions (m/z 326 and m/z 320, respectively).

Gas chromatograms of the fatty acid compositions from patient III-4 revealed that phytanic acid was found mainly in TG_2 and TG_1 (80% and 45%, respectively), while triunsaturated phytanic acid was associated with TG_1 and TG_0. Phytanic acid, but not triunsaturated phytanic acid, was also found in free fatty acids (5.5%) and phosphatidylcholine (13.5%). No branched chain fatty acids were found in cholesteryl esters. After fractionation by

centrifugation of plasma lipoproteins into different density subclasses, TG_1 and TG_2 were shown by HPTLC to be primarily localized in the LDL fraction (Fig. 5).

Approximately 30–60 mg/dl of phytanic acid and 5–9 mg/dl of triunsaturated phytanic acid were found in the plasma of affected patients (Table 1). The concentration of the major plasma fatty acids (16:0, 18:0, 18:1, 18:2 and 20:4) was not changed substantially by the accumulation of these branched chain fatty acids (Table 1). To further test whether the acylation process is affected by the presence of branched-chain fatty acids, the activity of plasma LCAT was compared between affected patients and control subjects. No significant differences were found in either the rate of cholesterol esterification or the fatty acid composition of radiolabeled cholesteryl esters formed by the LCAT reaction (data not shown).

Erythrocyte lipids. Both saturated and unsaturated phytanic acids were found in triacylglycerols isolated from affected patients (Table 2). The increase of branched chain fatty acids was correlated more closely with a decrease of 18:0, 18:1 and 20:4 and an increase of 18:2. The percentage distribution of 16:0 remained essentially unchanged. By HPTLC, the profile of neutral glycosphingolipids revealed that the ratio of hydroxy to nonhydroxy

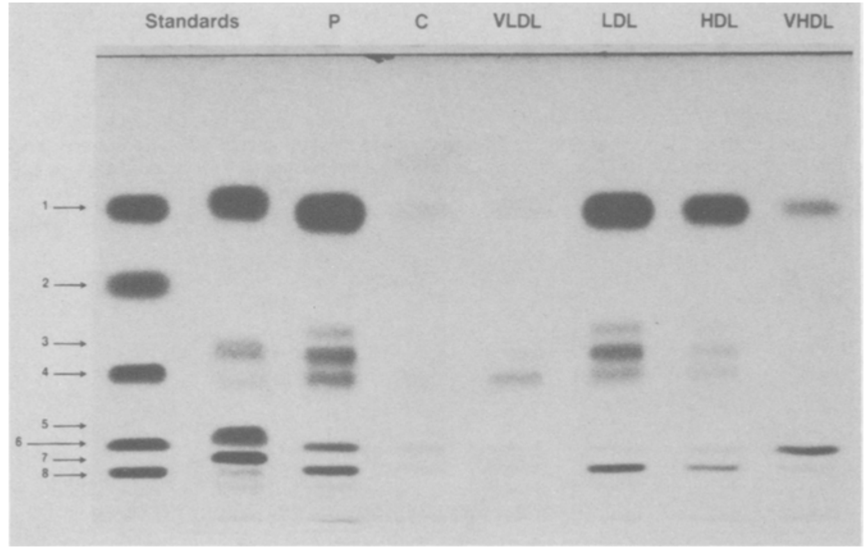

FIG. 5. HPTLC of nonpolar lipids from plasma and various lipoprotein fractions of patient III-4. P, plasma (0.01 ml); C, chylomicrons; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins, VHDL, very high density lipoproteins; 1–8, see legend of Fig. 2. Each lipoprotein fraction was separated from 0.02 ml of plasma.

TABLE 1

Major and Branched Chain Fatty Acid Contents (mg/dl) in Plasma of Normal Subjects and Patients with Refsum's Disease

Fatty acids	Normal controls (n = 5)	Affected			Unaffected		
		III-4	III-10	III-2	III-8	III-6	III-16
16:0	70 ± 6	64	72	73	70	109	72
16:1	5 ± 2	2	3	3	3	4	7
18:0	24 ± 4	21	25	26	30	36	33
18:1	73 ± 10	52	79	72	63	97	74
18:2	112 ± 13	89	128	151	113	129	152
20:3(n-6)	6 ± 1	3	5	5	4	7	8
20:4(n-6)	25 ± 8	23	25	29	19	34	53
22:4(n-6) + 24:1	2 ± 1	3	4	1	2	2	1
22:6(n-3)	4 ± 1	5	7	3	5	4	6
24:0	3 ± 1	2	4	3	3	3	3
16:0br[a]	—	62	30	46	—	—	—
16:3br[b]	—	9	5	7	—	—	—

[a]Phytanic acid.

[b]Triunsaturated phytanic acid (3,7,11,15-tetramethylhexadec-6,10,14-trienoic acid).

fatty acid moieties from affected patients was not substantially different from those of unaffected kins or control subjects (data not shown).

Biopsied sural nerve. The nonpolar lipids of endoneurium and the epi- and perineurium of affected (III-2 and III-10) and unaffected (III-6) patients were compared with their plasma nonpolar lipid profiles using HPTLC (Fig. 6). Both TG_1 and TG_2 bands were absent from endoneurial triacylglycerol isolated from the affected patients. Despite the presence of abundant triacylglycerols in epi- and perineurium, only a faint band of TG_1 appeared in the affected patients. The nonpolar lipids of interstitial

TABLE 2

Fatty Acid Composition of Erythrocyte Triacylglycerol

Fatty acids	Affected			Unaffected	
	III-2	III-4	III-10	III-6	III-16
14:0	1.25[a]	0.38	0.84	0.57	0.36
15:0	0.96	0.78	0.87	0.53	0.72
16:0	18.57	17.83	20.64	18.57	17.46
16:0br[b]	3.83	24.79	11.20	—	—
16:1	3.02	0.79	1.40	2.48	2.34
16:3br[c]	t[d]	2.19	1.13	—	—
18:0	10.37	5.86	8.59	11.88	12.39
18:1	37.36	21.73	30.96	41.32	42.82
18:2	7.52	13.11	10.16	8.59	7.01
20:0	0.58	0.45	0.48	0.45	0.45
20:1	0.73	0.56	0.80	0.86	0.86
20:2(n-6)	0.52	0.34	0.37	0.47	0.41
20:3(n-6)	0.60	t	0.44	0.99	1.00
20:4(n-6)	2.04	0.86	1.04	2.34	1.79
20:5(n-6)	1.39	0.84	0.79	0.89	0.55
22:4(n-6)	1.53	0.67	0.53	1.23	0.87
22:5(n-3)	0.34	—	0.51	0.49	0.46
22:6(n-3)	1.05	0.22	0.48	0.57	0.35
24:0	0.46	0.60	0.43	0.29	0.51

[a]The sum is less than 100% in each case because some minor and unidentified fatty acids were omitted from the final tabulations.

[b]Phytanic acid.

[c]Triunsaturated phytanic acid (3,7,11,15-tetramethylhexadec-6,10,14-trienoic acid).

[d]t, Trace amount.

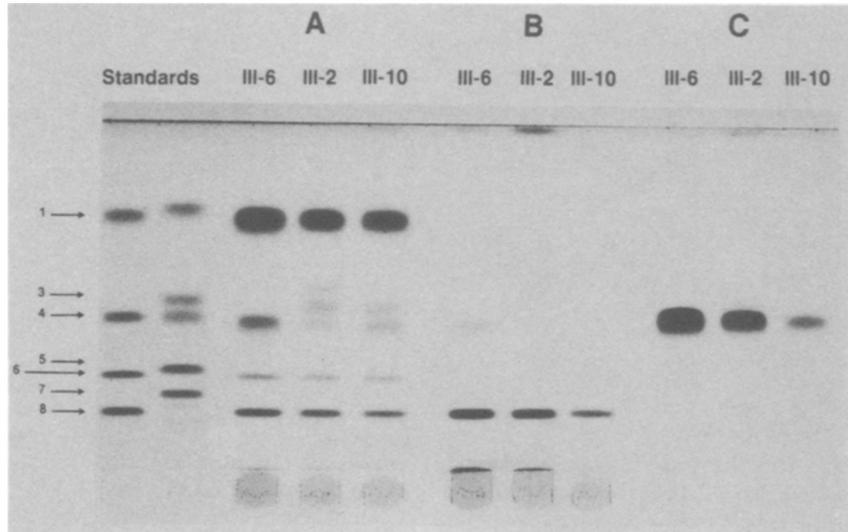

FIG. 6. HPTLC of nonpolar lipids. A, 5 μl plasma; B, 0.1 mg dry wt of biopsied sural endoneurium; C, 0.03 mg dry wt of biopsied sural epineurium and perineurium; 1–8, see legend of Fig. 2.

fat and blood vessels from affected (III-2) and unaffected (III-6) patients were also analyzed by HPTLC (data not shown). Like epi- and perineurium, only a faint band of TG_1 appeared in interstitial fats from the affected patient. There were no fast-moving bands of TG present in blood vessels from this patient.

The fatty acid composition of total endoneurial lipids (Table 3) and of epi- and perineurial triacylglycerols (Table 4) were further analyzed by capillary GC. In normal sural endoneurium, the major fatty acids were 16:0, 18:0 and 18:1. On the other hand, 16:0, 16:1, 18:1 and 18:2 were the major fatty acids in epi- and perineurial triacylglycerols. In affected patients, ca. 2% of the total fatty acids in endoneurium were found to be phytanate, which was probably associated with phospholipids. On the other hand, less than 5% of the total fatty acids in epi- and perineurial triacylglycerols were found to be phytanate. No branched chain fatty acids were demonstrated in either normal subjects or unaffected patients.

CSF. Analysis of CSF by HPTLC revealed that cholesteryl ester, unesterified cholesterol, phosphatidylcholine and sphingomyelin were the major lipid classes. To ascertain whether phytanate accumulation was present in CSF, the fatty acid compositions of cholesteryl ester and phospholipids were analyzed by capillary GC. The major fatty acids of normal CSF cholesteryl esters were 16:0, 18:1 and 18:2, whereas the major fatty acids of normal CSF phospholipids were 16:0, 18:0 and 18:1. In one of the affected patients, III-10, 0.9 μg of phytanate was found in 1 ml of CSF phospholipids. No phytanate was demonstrated in CSF cholesteryl esters.

DISCUSSION

The present study demonstrates that only small amounts of phytanic acid are present in desheathed sural nerves obtained by biopsy from three Refsum's patients with phytanic acid storage. This evidence makes it unlikely that phytanic acids are, in increased amounts, incorporated into the peripheral nerve myelin of affected patients. Thus, the molecular distortion hypothesis (11,12), in which incorporation into the cell membrane of branched chain fatty acids in place of the normal straight chain fatty acids resulting in membrane instability, seems unlikely as an explanation for the neuropathologic alterations. However, one possibility that cannot be excluded at this time is that the small amounts of phytanate might exert some damage on nerve fiber. Other observations also argue against the distortion hypothesis. First, as yet there is no evidence that nerve content of phytanic acid was influenced by a low-phytanate diet on which Refsum's patients were maintained (12). Second, only a low level of phytanic acid accumulated in the brain and nerves of animals fed large amounts of phytanic acid or its precursors (29,30), and neuropathy did not develop, although a low level of incorporation in the nervous system may be due to the serious toxicity of large doses of these branched chain fatty acids (12).

Accumulation of phytanic acid has been demonstrated in the sciatic nerve of Refsum's patients (11,31,32). These reports, however, did not specify whether phytanic acids were located in the myelin or endoneurium. It is conceivable that the accumulation of branched chain fatty acids in these previous cases was confined, as in this study, to the epi- and perineurium, which contains mainly

TABLE 3

Fatty Acid Composition of Endoneurial Lipids from Biopsied Sural Nerve

		Kinship members	
Fatty acids	Normal (n = 4)	Affected (n = 3)	Unaffected (n = 1)
16:0	20.3 ± 2.9[a]	17.5 ± 5.9	13.8
Phytanate	—	1.7 ± 0.8	—
17:0	1.0 ± 0.2	—	0.5
18:0	21.5 ± 4.7	13.1 ± 2.6	11.6
18:1(n-9)	20.3 ± 5.4	20.5 ± 0.9	21.2
18:1(n-11)[b]	1.9 ± 0.7	2.7 ± 0.5	2.8
18:2	3.3 ± 1.3	4.3 ± 2.0	3.4
20:0	2.2 ± 0.3	1.7 ± 0.3	1.6
20:1	2.3 ± 0.3	2.2 ± 1.2	3.1
20:4(n-6)	1.9 ± 0.5	3.4 ± 1.3	3.3
22:0	2.9 ± 0.5	2.8 ± 0.2	2.7
22:4(n-6)	1.6 ± 0.5	2.0 ± 1.1	2.1
23:0	1.2 ± 0.4	1.4 ± 0.5	1.8
24:0	2.4 ± 1.2	4.3 ± 1.2	4.6
24:1(n-9)	4.9 ± 0.9	5.6 ± 3.1	7.4

[a]Values are expressed as mean and S.D. The sum is less than 100% in each case because 14 minor (14:0, 15:0, 16:1, 18:1 *trans*, 19:0, 21:0, 20:2(n-6), 20:3(n-6), 22:1, 22:5(n-3), 22:6(n-3), 23:1, 25:0 and 26:0; each <1%) and nine unidentified fatty acids (5-10%) were omitted from the final tabulations.

[b]Tentative identification.

TABLE 4

Fatty Acid Composition of Triacylglycerol in Epineurium and Perineurium of Biopsied Sural Nerve

		Kinship members	
Fatty acids	Control (n = 4)	Affected (n = 3)	Unaffected (n = 1)
14:0	0.8 ± 0.2[a]	1.5 ± 0.1	1.4
16:0	13.7 ± 1.9	14.5 ± 1.3	13.7
16:1	10.1 ± 2.9	10.1 ± 4.3	10.5
Phytanate	—	3.7 ± 0.6	—
18:0	0.7 ± 0.2	1.4 ± 0.5	1.1
18:1(n-9)	46.5 ± 2.3	42.9 ± 5.1	48.8
18:1(n-11)[b]	5.9 ± 1.1	5.9 ± 3.9	2.7
18:2(n-6)	13.6 ± 1.9	11.0 ± 3.4	11.4
18:3(n-3)	0.5 ± 0.1	0.4 ± 0.1	0.5
19:0	0.6 ± 0.1	0.4 ± 0.2	0.6
20:1	1.6 ± 0.1	1.3 ± 0.1	1.1
20:2(n-6)	0.5 ± 0.1	0.3 ± 0.0	0.2
20:3(n-6)	0.2 ± 0.0	0.2 ± 0.1	0.3
20:4(n-6)	0.9 ± 0.1	0.7 ± 0.1	1.1
22:4(n-6)	0.7 ± 0.2	0.5 ± 0.1	0.4
22:5(n-3)	0.2 ± 0.0	0.2 ± 0.1	0.3
22:6(n-3)	0.2 ± 0.1	0.2 ± 0.1	0.2

[a]Values are mean and S.D. The sum is less than 100% in each case because three minor (15:0, 18:1 *trans* and 20:0) and eight unidentified fatty acids were omitted from the final tabulations.

[b]Tentative identification.

triacylglycerol (Fig. 6). Because the metabolic (33,34) and compositional (35) differences exist between the endoneurial and epi- and perineurial tissues, it is essential to utilize the endoneurium (desheathed nerve) in the study of the metabolic derangements of peripheral nerve lipids.

Laurell (36) has suggested that a relative deficiency of linoleate may be induced by the accumulation of phytanic acid. His speculation was based primarily on the analysis of plasma triacylglycerol fatty acid composition. However, most of the linoleic acid is normally found in plasma cholesteryl esters and phospholipids. Our data on the fatty acid concentrations of plasma total lipids (Table 1) indicate that the absolute concentration of linoleate and arachidonate are not affected by the presence of branched chain fatty acids. On the other hand, displacement of linoleate and arachidonate has been demonstrated in the phospholipids of liver, heart, kidney and skin (32,37). Thus, it is possible that the utilization of essential fatty acids is abnormal in the presence of branched chain fatty acids.

Two novel branched chain fatty acids, mono- and triunsaturated analogues of phytanic acid, were first demonstrated by Dulaney et al. (38) in the serum and urine of patients with Refsum's disease. Later, Evans and Dulaney (39) identified these unsaturated phytanic acids as 3,7,11,15-tetramethylhexadec-15-monoenoic acid and 3,7,11,15-tetramethylhexadec-6,10,14-trienoic acid. In the present study, we have also been able to demonstrate the presence of triunsaturated phytanate in the plasma (Table 1 and Fig. 3) and erythrocytes (Table 2) of affected patients. The level of these unsaturated analogues is probably proportional to the amount of accumulated phytanic acid. Steinberg (12) has suggested that triunsaturated phytanate may represent a metabolite of geranylgeranyl pyrophosphate. However, the origin of these unsaturated branched chain fatty acids remains speculative.

In conclusion, lack of phytanate accumulation in peripheral nerves, even in cases with severe fiber loss, raises significant doubt that the stability of peripheral nerve myelin, altered by the incorporation of branched chain fatty acids into myelin, is the basis of the neuropathologic alteration. Future studies need to critically assess whether Schwann cells (myelin) or neurons (axons) are preferentially affected and whether demyelination is primary or secondary.

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Ether Glycerophospholipids of Gills of Two Pacific Crabs, *Cancer antennarius* and *Portunus xantusi*

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Phospholipids and sterols constituted 70 and 20%, respectively, of the total lipids of the gills of two crabs, *Cancer antennarius* and *Portunus xantusi*. Phosphatidylcholine (46–55% of the total phospholipid phosphorous) and phosphatidylethanolamine (24–25%) were the principal phospholipids present. In both species 1′-alkenyl glycerols were present in about 20% of the phospholipid molecules but were not detected in the neutral gill lipids. The total ether phospholipids of *C. antennarius* gills contained 62% 1-(1′-alkenyl) groups, with the remainder probably being 1-alkyl moieties. Total gill plasmalogen contents were in the range of 163–184 μmol/g lipid, 82–87% of which was in the phosphatidylethanolamine fraction in both crab species.
Lipids 22, 76–79 (1987).

The gill lamellae of crustaceans are exposed to the external aquatic medium; thus their gill membranes, which contain high amounts of phospholipids (1–3), may be directly affected by the environment (4–6). Consequently, the study of gill phospholipids may help us to understand the role of lipids in environmental adaptations. The phospholipids of crustacean gills have been studied in some detail (7), but little information is available regarding the presence of ether-containing phospholipids, i.e., alkylacyl and 1-(1′-alkenyl)-2-acyl (plasmalogen) phosphoglycerides. The plasmalogen levels in gills isolated from various species of aquatic animals have been studied in several laboratories (8–11). Nothing is known about the existence of neutral plasmalogens (1′-alkenyl-diacyl glycerols) or of alkylacyl-phosphoglycerols in gills of marine crustaceans.

Ether lipids appear to be important constituents of many animal cell membranes (12), and some of the functions proposed for this lipid type in membranes have been reviewed briefly by Arthur et al. (13). As background to future study of the role of gill ether lipids in the adaptation of aquatic animals to environmental changes, we quantified the lipid composition and investigated the amounts of alkyl and 1′-alkenyl moieties in the gill lipids of two crabs, *Cancer antennarius* and *Portunus xantusi*, from the southern California coast.

MATERIALS AND METHODS

The large crab *Cancer antennarius* and the swimming crab *Portunus xantusi* were collected locally, and tissues (gills, hepatopancreas and muscle) were rapidly isolated and weighed. Lipid extraction and phospholipid determination by phosphorus analysis were performed as described previously (1,2). Total plasmalogens were measured by iodine uptake according to the procedure of Williams et al. (14), and assays of the content of choline and ethanolamine plasmalogens were carried out as described previously (11).

Total ether-linked glycerolipids were determined quantitatively by the method of Blank et al. (15): a) a 2-mg aliquot of the solvent-free total lipids was dissolved in 2 ml of diethyl ether and vigorously mixed with 0.7 ml of 0.05 M phosphate buffer (pH 7.1) containing about 3 units of phospholipase C from *Bacillus cereus* (Sigma Chemical Co., St. Louis, Missouri). After 2.5 hr at room temperature, the diethyl ether was evaporated under nitrogen, and the lipids were saponified with 2.5 ml of 0.5 M KOH in methanol in a boiling water bath for 8 min to remove acyl groups. Finally, 4.5 ml of methanol/acetic acid (100:6, v/v), 7 ml of chloroform and 6 ml of water were added. The upper phase was extracted again with 7 ml of chloroform, and the combined chloroform extracts were evaporated to dryness under vacuum. The total alkyl and 1′-alkenyl ether contents of the glycerolipids were determined from the aldehydes produced by the oxidation of the glycerol chains: b) the phospholipase C/saponification products from 1 mg of total lipids were treated with 0.5 ml of 0.1 M sodium metaperiodate in 90% acetic acid and heated for 45 min at 50 C. c) A second 1-mg sample of the products from procedure a was dissolved in 0.5 ml of 90% acetic acid containing 0.5 M HCl and heated for 45 min at 50 C to release aldehydes from the 1′-alkenylglycerols. Reaction mixtures from steps b and c were cooled to room temperature and ca. 150 mg of Silica Gel G (E. Merck, Brinkmann Instruments Co., Westbury, Connecticut) was added to remove compounds interfering with the color development. The aldehydes formed were measured at 546 nm after reaction with fuchsin reagent as described by Gray (16). The amounts of 1′-alkenylglycerols (directly) and alkylglycerols (by difference) were calculated from a standard curve determined with 1-*O*-hexadecyl-*rac*-glycerol (Sigma) over the range of 0–30 μg. Note that the mixture of reaction products assayed in steps b and c would also include glyceryl ethers from any neutral diacylglyceryl ethers and/or 1-(1′-alkenyl)-2,3-diacyl glycerols present.

Hydrolysis of the phospholipids was monitored on thin layer chromatograms using Phospray (Supelco, Bellefonte, Pennsylvania) for the detection of lipid phosphorous. To detect and separate alkylglycerols and 1′-alkenylglycerols, the unsaponifiables of total, neutral and phospholipid fractions from *C. antennarius* gills were separated by one-dimensional thin layer chromatography (TLC) using petroleum ether/diethyl ether/acetic acid (30:70:1, v/v/v) on Silica Gel G (17). The spots were located by spraying with 3% cupric acetate in 8% aqueous phosphoric acid (18) and careful charring; they were identified by comparison with authentic standards of cholesterol, 1-hexadecylglycerol, ceramide (obtained from sphingomyelin by the phospholipase C/saponification technique) and 1′-alkenylglycerols (from beef heart phospholipids, rich in plasmalogens [19], by the same technique).

An aliquot of the total lipids of *C. antennarius* gills was fractionated by column chromatography on silicic acid (Bio-Rad A, Bio-Rad Laboratories, Richmond, California)

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into neutral lipids (eluted with 4 column vol of chloroform), glycolipids (collected in 4 vol of acetone) and the phospholipids (eluted last with 3 vol methanol) (20). Quantitative analysis of lipids was also accomplished with the Iatroscan TLC/FID (model TH-10, Mk. II) according to the procedure of Hakanson (21) using SI Chromarods. We spotted 20 μg total lipid in 1 or 2 μl of n-hexane. The rods were developed with hexane/diethyl ether/acetic acid (93:7:0.3, v/v/v) and scanned through the FID at 32 sec/rod; the hydrogen pressure was 1.2 kg/cm^2 and the air flow 2 l/min. From the electronically integrated FID signal the different lipid types were quantified using calibration curves for known compounds.

RESULTS

Table 1 presents the distribution of different lipids found in gills of crabs *Cancer antennarius* and *Portunus xantusi*. The principal lipids of both crustaceans are phospholipids (69–70% of total lipids) and sterols (18–21%, probably largely cholesterol [1,22]). Some minor components also occur, such as triglycerides, free fatty acids and cholesteryl esters; glycolipids were about 10% of total lipids by silicic acid column chromatography. The gill lipid composition of *P. xantusi* closely resembled that of *C. antennarius*.

Table 2 gives the amount of plasmalogens in gills, hepatopancreas and muscle of the two crabs. It is clear that the highest plasmalogen contents in both crabs were detected in gills (17.8–20.6%).

The composition of different classes of phospholipids isolated from gills (Table 3) is generally very similar in both species of Pacific crabs. The principal gill phospholipids are phosphatidylcholine (mainly diacyl forms) and phosphatidylethanolamine (essentially in the plasmalogenic form). This tissue is also rich in sphingomyelin and diphosphatidylglycerol.

TLC of the unsaponifiables of the gill total lipids from *C. antennarius* gave a distinct spot for 1′-alkenylglycerols with an approximate Rf value of 0.59 under our experimental conditions; this is higher than the Rf of 1-hexadecylglyceryl ether (0.42). Both alkyl and 1′-alkenyl ethers were found in the phospholipids, but the neutral lipids of gills contained only traces of alkylglycerols.

It was of interest to determine the total content of glyceryl ethers in gill phospholipids. The results for *C. antennarius* only are summarized in Table 4 and reveal a high concentration of ether lipids (16–21% of total phospholipids). When the fuchsin reagent was used, the values for plasmalogens (1.30 mg/g wet tissue) were not significantly different from the results obtained with the iodine uptake procedure (Table 2; 1.46 mg/g wet tissue).

TABLE 1

Composition of Principal Lipids of Gills Obtained from Crabs *C. antennarius* and *P. xantusi*

Species	Total lipids (mg/g wet wt)	Total phospholipids[a] mg/g wet wt	Total phospholipids[a] Percent of total lipids	Percent of total lipids[b] Sterol	Percent of total lipids[b] Triglycerides	Percent of total lipids[b] Fatty acids	Percent of total lipids[b] Unknown[c]
C. antennarius	10.3 ± 1.8 (n = 8)[d]	7.1 ± 1.2 (n = 8)	68.9	18.5	2.2 (n=3)	1.5	2.9
P. xantusi	10.7 ± 1.6 (n = 3)	7.5 ± 0.8 (n = 5)	70.0	21.2	1.4 (n = 2)	2.2	No data

[a]By quantitation of lipid phosphorous using the factor: lipid phosphorous = 4.0% wt of total phospholipids (2).
[b]Analyzed by Iatroscan TLC-FID. For *C. antennarius*, the total phospholipids (including glycolipids) were 75% of total lipids. By column chromatography (see Materials and Methods), the total lipids consisted of 21% neutral lipids, 69% phospholipids and only 10% of glycolipids.
[c]Unidentified compounds: principally cholesteryl ester.
[d]Results are given as the mean of n crabs.

TABLE 2

Concentrations of Plasmalogens in Various Tissues of Crabs *C. antennarius* and *P. xantusi*

	C. antennarius			*P. xantusi*	
	Gills (n = 11)	Hepatopancreas (n = 1)	Muscle (n = 2)	Gills (n = 5)	Muscle (n = 1)
μmol/g lipid	184.1 ± 16.4	55.9	93.4	162.6 ± 19.2	46.7
mg/g wet wt[a]	1.46 ± 0.26	3.9	0.55	1.34 ± 0.21	0.34
Wt percent of total lipids	14.1	4.3	7.2	12.5	3.6
Wt percent of total phospholipids	20.6	—	—	17.8	6.4

Results are given as the mean of n crabs and are determined by the iodine-uptake method of Williams et al. (14).
[a]Assuming mol wt of 770 (10).

TABLE 3

Phospholipid Composition in Gills of *C. antennarius* and *P. xantusi* (% of Total Phospholipid Phosphorous Content)

Phospholipid class	*C. antennarius* (n = 4)	*P. xantusi* (n = 2)
Phosphatidylcholine (PC)	54.8 ± 7.8	46.5
Diacyl + alkylacyl	52.7	43.7
Alkenylacyl (CP)	2.1 ± 1.1	2.8
Phosphatidylethanolamine (PE)	24.8 ± 2.6	24.1
Diacyl + alkylacyl	11.3	11.2
Alkenylacyl (EP)	13.6 ± 1.8	12.9
Sphingomyelin	7.4 ± 0.9	12.0
Phosphatidylserine	2.3 ± 0.3	2.5
Phosphatidylinositol	1.9 ± 0.3	3.1
Diphosphatidylglycerol	3.1 ± 0.5	7.7
Phosphatidic acid	2.6 ± 0.6	2.5
	Ratio %	
CP/PC	3.8	6.0
EP/PE	54.8	53.5
CP/(CP + EP)	13.4	17.8
EP/(CP + EP)	86.6	82.2

TABLE 4

Alkylglycerols and 1′-Alkenylglycerols of *C. antennarius* Gills

	mg/g Fresh wt		Ether glycerols Wt % of total	Phospholipids (Wt % of total)		
Ether glycerols						
Total	nd	2.08 = 0.23[a]	(100%)[a]	nd	29.3%[a]	nd
1′-Alkenyl	1.46 = 0.26[b]	1.30 = 0.15	62.3	20.6[b]	18.3	15.7%[c]
Alkyl (by difference)	nd	0.78 = 0.12	37.5	nd	11.0	nd

nd, No data.

[a]Method of Blank et al. (15); total ether glycerols of total lipids. Assumes mol wt of 770 for in situ plasmalogens (10). Values are the mean of analyses of six individual animals.

[b]Method of Williams et al. (14); total 1′-alkenyl moieties of total lipids. Data of Tables 1 and 2.

[c]Method of Chapelle and Benson (11); choline plus ethanolamine plasmalogens only.

The alkylglycerol content (obtained by difference between total ether lipids and 1′-alkenyl forms) was also substantial, being about 0.78 mg/g of wet tissue, 11% of total phospholipids or 37.5% of the total ether lipids.

DISCUSSION

This investigation confirms that marine crustacean gills are a rich source of ether lipids (about 29% of total phospholipids in *C. antennarius*). Crab gills for the most part have phosphatidylethanolamines as principal plasmalogens (82.2–86.6% of total 1′-alkenylphosphoglycerols; Table 3). This agrees with our previously published data on gill plasmalogens for several different crustaceans (11). Phosphatidylethanolamine has been reported to be the principal plasmalogen in whole body lipids of arthropoda and many other marine invertebrates (8,23). We found no neutral plasmalogens ([1′-alkenyl]-diacylglycerols) in the gill lipids of *C. antennarius,* but estimate 1-alkyl-2-acyl phosphoglycerols to be about 11% of the phospholipids (Table 4).

1-Alkyl glyceryl ethers are widely distributed in marine organisms (8,23,24), but their occurrence as constituents of the phospholipids (i.e., as 1-alkyl-2-acyl-3-phosphoglycerols) is not well documented. The occurrence of neutral plasmalogens ([1′-alkenyl]-2,3-diacylglycerols) has been reported only for starfish (24,25) and ratfish liver (17).

Together with the literature, our results lead to the conclusions that in crustacean gills the phospholipids, although important sources of both alkyl and 1′-alkenyl ether phospholipids, contain more 1′-alkenyl than alkyl ether moieties and that neutral plasmalogens (i.e., [1′-alkenyl]-2,3-diacylglycerols) seem to be absent. The levels of plasmalogens in gill lipids of crustaceans are comparable to the content of these plasmalogenic substances in the lipids of mammalian heart and brain, with the latter

tissues being considered the most concentrated sources in vertebrates.

Extensive data document the role of gill membrane phospholipids and fatty acids in adaptation of aquatic invertebrates and vertebrates to environmental and salinity changes (7,26). We believe that similar studies of the response of gill membrane ether phospholipids (changes in the amounts of specific phospholipids and ether glycerophosphates and in the carbon chains of their fatty acids and 1-alkyl and 1′-alkenyl moieties) could offer additional parameters by which to investigate the role of ether phospholipids in biological systems.

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Free and Esterified Sterols of Cotton Buds and Anthers

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Capillary gas liquid chromatography analyses were conducted on free and esterified sterol fractions of cotton (*Gossypium hirsutum* cv. Stoneville 213) floral buds and anthers. The free sterols of both cotton buds and anthers consist mainly of the common plant sterols sitosterol, stigmasterol and 24ξ-methylcholest-5-en-3β-ol. The composition of esterified sterols of cotton buds and anthers were similar, and consisted of pollinastanol, 31-norcycloartanol, cycloartenol, 31-norcycloartenol, 24-dehydropollinastanol and sitosterol. Desmosterol was also present in both the free and esterified sterols of anthers. The identities of the sterols were confirmed by gas chromatography–mass spectrometry analyses. Esterified sterols accounted for 46.7 and 88.7% of total sterols of cotton bud and anthers, respectively. The ratio of esterified sterol to free sterol per gram of tissue is about 8:1 in anthers. The Δ^5-sterols of the esterified sterols of cotton buds and anthers account for only 17 and 9.2% of the total sterols, respectively.
Lipids 22, 80–83 (1987).

The wax-sterol ester fraction of freeze-dried cotton buds or anthers has exhibited potent feeding stimulant activity for the cotton boll weevil (1). While demonstrating that certain waxes of the wax-sterol ester mixture were responsible for the feeding activity (1), it was observed that the composition of esterified sterols differed qualitatively and quantitatively from that of the free sterols and from results of a previous report (2). Most phytophagous insects are capable of converting the ubiquitous plant sterol sitosterol, as well as a variety of Δ^5-C_{28} and -C_{29} plant sterols, to cholesterol to obtain adequate quantities of this "essential" sterol (3). The fact that the boll weevil is capable of converting sitosterol, a major sterol of cotton buds, to cholesterol (4) and is stimulated to feed on and oviposit in cotton buds demonstrates an important host plant–insect relationship and the essential role of cotton buds in the life cycle of the cotton boll weevil. The composition of the esterified sterols suggests that the cotton boll weevil depends on the free sterols of cotton buds or anthers to satisfy its metabolic needs for sterols. The present paper describes the composition and identity of the free and esterified sterols of cotton buds and anthers.

MATERIALS AND METHODS

Instrumentation. Sterols and sterol acetates were analyzed by gas liquid chromatography (GLC). The GLC analyses were performed with a Varian model 3700 gas chromatograph equipped with a flame ionization detector and a 15 m × 0.32 mm DB-1 fused silica capillary column (0.25 μm film) at oven temperatures of 230 C and 240 C for free sterols and sterol acetates, respectively. Peaks, areas of peaks and retention times were automatically recorded by a Shimadzu model C-R1B recorder. Cholesterol, sitosterol and their acetates were used as standards. Electron ionization (70 eV) mass spectra were obtained with a Finnigan model 4500 gas chromatograph–mass spectrometer fitted with a 15 m × 0.25 mm DB-1 fused silica capillary column (0.1 μm film thickness). Data were analyzed by an on-line Incos data system. Thin layer chromatography (TLC) analyses were conducted on Anasil G Chromatoplates (Analabs, North Haven, Connecticut). Silver nitrate-impregnated Silica Gel H Chromatoplates (Analabs) were prepared as previously reported (5). Visualization of TLC spots was achieved by spraying with sulfuric acid followed by heating.

Extraction of cotton buds and anthers. Whole cotton (*Gossypium hirsutum* cv. Stoneville 213) floral buds with bracts were chopped in a blender and then freeze-dried. Anthers were dissected from buds and then freeze-dried whole. Twenty-five g of each freeze-dried material was extracted in a Soxhlet apparatus successively for 24 hr each with pentane, ethyl acetate, chloroform and methanol (1). The solvents were removed under reduced pressure at 50 C, and the resulting residues were analyzed by TLC on two separate plates; one was developed in toluene/hexane (1:1, v/v) and the other in toluene/ethyl acetate (9:1, v/v).

Free sterol and sterol ester isolation. After Soxhlet extraction, TLC analyses showed that the major portions of the unesterified sterols (free sterols) and wax-sterol ester mixtures were in the pentane extract with small quantities of each in the ethyl acetate extract. To remove the wax-sterol ester mixture and free sterols from more polar compounds in the ethyl acetate extract, the residue was partitioned between equal volumes of pre-equilibrated hexane and 70% aqueous methanol. The lower phase (70% aqueous methanol) was passed through a total of five funnels containing the hexane phase.

The hexane phase was washed twice with the lower phase. TLC analyses showed that there were neither free sterols nor sterol esters in the lower phase (70% methanol), and it was discarded. The combined upper phases, which contained the wax-sterol ester mixture and free sterols, were reduced to dryness under vacuum and combined with the pentane extract. The combined residues were chromatographed over 30 g of hexane-washed Merck Silica Gel (2.5 × 16 cm) by eluting with 150-ml volumes of 0 and 3% ether in hexane, with 100-ml volumes of 10, 25 and 50% ether in hexane, and finally with 100% ether. The wax-sterol ester mixture was present in the 3% ether in hexane fraction. The 25% and 50% ether-in-hexane fractions that contained a mixture of alcohols, sterols, diglycerides and fatty acids were combined.

Removal of fatty acids from sterol mixture. The combined 25% and 50% ether-in-hexane fractions were

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Nomenclature—sitosterol: 24α-ethylcholest-5-en-3β-ol; 24ξ-methylcholest-5-en-3β-ol; stigmasterol: 24α-ethylcholesta-5,22E-dien-3β-ol; desmosterol: cholesta-5,24-dien-3β-ol; pollinastanol: 9β,19-cyclo-14α-methyl-5α-cholestan-3β-ol; 24-dehydropollinastanol: 9β,19-cyclo-14α-methyl-5α-cholest-24-en-3β-ol; cycloartenol: 9β,19-cyclo-4,4,14α-trimethyl-5α-cholest-24-en-3β-ol; 31-norcycloartanol: 9β,19-cyclo-4α-14α-dimethyl-5α-cholestan-3β-ol; 31-norcycloartenol: 9β,19-cyclo-4α,14α-dimethyl-5α-cholest-24-en-3β-ol; 24-methylenecholesterol: 24-methylcholesta-5,24(28)-dien-3β-ol.

reduced to dryness under vacuum, redissolved in ether and treated with an excess of diazomethane and a catalytic amount of methanol to esterify fatty acids. After the solvent was removed under vacuum, the residue was chromatographed on a silica gel column as before, and the fractions were monitored by TLC. The 10% ether-in-hexane fraction contained the fatty acid methyl esters. The 25% and 50% ether-in-hexane fractions containing the alcohols, sterols and diglycerides were combined and analyzed qualitatively and quantitatively by GLC.

Sterols from wax-sterol ester mixture. The 3% ether-in-hexane fraction, which contained the wax-sterol ester mixture, was saponified by refluxing with 5% potassium hydroxide in methanol/benzene (5:1, v/v) for 4 hr. Most of the solvent was removed under vacuum, and the mixture was diluted with water, acidified with dilute hydrochloric acid, and extracted three times with hexane. The hexane solution was washed with water and dried over sodium sulfate, and the hexane was removed under vacuum. The dried residue was dissolved in ether and treated with excess of diazomethane to esterify fatty acids. The ether was removed under vacuum and the residue was chromatographed over 30 g of silica gel (2.5 × 16 cm) as before in the removal of fatty acids from the sterol mixture. The column fractions were monitored by TLC.

Acetylation and argentation chromatography of esterified sterols. Since the compositions of the esterified sterols (from saponification of the wax-sterol ester fraction) from cotton buds and anthers were similar, they were combined and acetylated with acetic anhydride-pyridine overnight at room temperature. The sterol acetates were chromatographed on a 20% $AgNO_3$-Unisil column (1.5 × 27 cm), which was eluted with 50 ml of hexane followed by 50-ml volumes containing increasing amounts of ether in hexane (0.5 to 6.0%, in 0.5% steps). The fractions were monitored by GLC and TLC ($AgNO_3$-silica gel).

RESULTS

Free sterols from cotton buds and anthers. Extraction of cotton buds and anthers using successive solvents in a Soxhlet apparatus rather than the usual method, for example, with chloroform/methanol (2:1, v/v), yielded fractions that were less contaminated with more polar material. Both sterol esters and free sterols were completely extracted into pentane and ethyl acetate. The TLC and GLC analyses indicated that the 25% and 50% ether-in-hexane fractions from chromatography of the combined pentane and ethyl acetate extracts of both cotton buds and anthers, after removal of fatty acids, contained a mixture of sterols, a small quantity of other alcohols and (compared to our experiences with that of insect and plant tissues) an unusually large quantity of diglycerides. From 25 g of freeze-dried cotton buds, 53 mg of the alcohol-diglyceride-sterol mixture was obtained. The analysis of the mixture of sterols by GLC indicated a total of ca. 35 mg of sterols consisting mainly of sitosterol (87%) with lesser amounts of stigmasterol, 24ξ-methylcholest-5-en-3β-ol and detectable quantities of pollinasterol and cholesterol (Table 1).

Analogous fractions from 25 g of cotton anthers yielded 130 mg of the alcohol-diglyceride-sterol mixture, of which 32 mg was sterols. The free sterols of cotton anthers also consisted predominantly of sitosterol (78%) with lesser amounts of 24ξ-methylcholest-5-en-3β-ol, cholesterol, pollinastanol, stigmasterol and a detectable quantity of desmosterol (Table 1). The relative retention times (RRTs) (Table 1) and mass spectral data of the sterols were identical to those of authentic sterol standards.

Although column chromatography on silica gel did not separate the free sterols from the diglycerides, they were readily separated as their acetates. Acetylation of the sterol-diglyceride mixture followed by chromatography over 10 g of silica gel (2.5 × 6 cm) yielded the crystalline

TABLE 1

Relative Percentages of Total Free and Esterified Sterols of Cotton Buds and Anthers

		Cotton buds		Cotton anthers	
Sterols	RRT[a]	Free	Esterified	Free	Esterified
Sitosterol	1.65	87.0	17.0	78.4	5.7
Stigmasterol	1.42	7.0	—	3.6	—
24ξ-methylcholest-5-en-3β-ol	1.30	5.0	—	8.4	—
Pollinastanol	1.16	D[b]	26.0	4.3	29.5
Cholesterol	1.00	D	—	5.3	—
Cycloartenol	1.90 (1.77)	—	22.0	—	19.0
31-Norcycloartenol	1.51 (1.46)	—	16.0	—	17.6
31-Norcycloartanol	1.38 (1.34)	—	14.0	—	20.0
24-Dehydropollinastanol	1.28	—	5.0	—	4.0
Desmosterol	1.10	—	—	D	3.5
24-Methylenecholesterol	1.27	D	—	D	—
% of total sterol		53	47	11	89

[a]Gas liquid chromatographic relative retention times, expressed relative to cholesterol on DB-1. Values in parentheses are for sterol acetates relative to cholesterol acetate. When no value is given, it is the same as the free sterol value.

[b]Detectable quantity.

sterol acetates in the 2% ether-in-hexane fraction; the diglyceride acetates were eluted from the column with 20% ether in hexane. TLC analyses and gravimetric quantitation of the acetate fractions from cotton anthers indicated 32 mg of sterol acetates and 81 mg of the diglyceride acetates; from cotton buds, 32 mg of sterol acetates and 14 mg of diglyceride acetates were obtained.

Interestingly, like triglycerides, the diglyceride acetates (R_f = 0.05) migrated only slightly from the origin on a TLC plate developed in toluene/hexane (1:1, v/v). The sterol acetates (R_f = 0.31), however, migrated nearly a third of the distance from the origin to the solvent front of the plate.

Esterified sterols from cotton buds and anthers. Saponification of the wax-sterol ester fractions isolated from cotton buds and anthers yielded about 85% sterols and 15% alcohols, which consisted of phytol, geranylgeraniol, dihydrophytol and a C_{18} alcohol with a double bond (1). The alcohols and sterols were eluted in the 25% and 50% ether-in-hexane fractions from a silica gel column. The 25% ether-in-hexane fraction showed two spots by TLC with R_f values of 0.25 and 0.31, which were higher than that of a 4-desmethyl sterol (R_f = 0.17). The 50% ether-in-hexane fraction showed two spots with R_f values of 0.18 (similar to that of a 4-desmethyl sterol) and 0.26. Since the aliphatic alcohols and the 4-methyl and 4,4-dimethyl sterols chromatographed similarly, to determine the total sterol composition the 25% and 50% ether-in-hexane fractions were combined and analyzed qualitatively and quantitatively by GLC. Thus, 25 g of freeze-dried cotton buds yielded 55 mg of semicrystalline material, of which 31 mg were sterols by GLC analysis. The common plant sterols sitosterol and 24ξ-methylcholest-5-en-3β-ol represented only 17% of the total sterols (Table 1). A far greater quantity of the wax-sterol ester mixture was obtained from 25 g of freeze-dried cotton anthers than from the same weight of cotton buds. The mixture from anthers, when quantified by GLC, contained 250 mg of sterols, which consisted of 4,4-dimethyl, 4-methyl and 4-desmethyl sterols (Table 1).

The compositions of esterified sterols from cotton anthers and buds were quite similar and consisted mainly of pollinastanol (1), 31-norcycloartanol (2), cycloartenol (5), 31-norcycloartenol (4), 24-dehydropollinastanol (3) (Scheme 1; Table 1) and sitosterol. (Previous investigations on higher plant sterols have shown that while 24-ethylcholest-5-en-3β-ol has the 24α-configuration, the 24-methylcholest-5-en-3β-ol is often a mixture of 24α- and 24β-epimers [6] and thus the 24ξ-methyl designation for the latter compound.)

(1) R = R' = H
(2) R = Me, R' = H

(3) R = R' = H
(4) R = Me, R' = H
(5) R = R' = Me

SCHEME 1

In a previous study of the esterified sterols of cotton buds, cholesterol and sitosterol reportedly were found (2). In this study, however, the above-named sterols are the major esterified sterols of cotton buds and anthers, and sitosterol constitutes less than 6% of the total esterified sterols (Table 1). The esterified sterols of cotton anthers also contained demosterol. The sterol RRTs (Table 1) and mass spectral data were identical to those of authentic sterol standards.

With few exceptions, the RRT of a sterol vs cholesterol is identical to that of the corresponding sterol acetate vs cholesterol acetate (7,8). The exceptions are sterols with one or two methyl groups at C-4. For example, on an SE-30 column, the acetates of 31-norcycloartanol and cycloartenol gave RRTs of 1.35 and 1.75, respectively. The corresponding free sterols exhibited RRTs of 1.39 and 1.87, respectively (7,8). Surprisingly, on our 15-m DB-1 (a crosslinked and bonded dimethylpolysiloxane liquid phase) fused silica capillary column, the RRTs for the acetates of 31-norcycloartanol and cycloartenol were 1.34 and 1.77, respectively; the free sterol RRTs were 1.38 and 1.90, respectively (Table 1) and thus agree well with the RRTs obtained on an SE-30 coated column (7,8). The RRTs of the 4-desmethyl sterols were also similar to those previously reported (7,8). In order to have the RRT of a sterol vs cholesterol identical to that of a sterol acetate vs cholesterol acetate, the column temperature for analyses of the sterol acetate should not be more than 10 C higher than the column temperature for the free sterol.

Although 4-desmethyl sterols accounted for nearly 43% of the total esterified sterols from cotton anthers, the common 4-desmethyl sterols such as sitosterol and stigmasterol represented less than 6% of the total sterols. The composition of esterified sterols is quite similar in both buds and anthers, but the total quantity of sterols per gram of tissue is about eight times greater in anthers.

Argentation chromatography of the sterol acetates from the combined esterified sterols of cotton buds and anthers, followed by qualitative GLC analyses, was effective in separating the sterols on the basis of degree of unsaturation (Table 2). Fractions 5 and 6 contain the saturated steroids. Fractions 7–9 contain the steroids with one double bond whereas fraction 10 contains those with two double bonds. Since 4,4-dimethyl, 4-methyl and 4-desmethyl sterols are readily separated by chromatography over neutral alumina (8,9), it would be possible to obtain each of the steroids in Table 2 in high purity simply by saponification of the saturated and mono- and diunsaturated steroid fractions and subsequent chromatography on an alumina column. In fact, the argentation chromatography shows some degree of separation within the saturated, mono- and diunsaturated fractions on the basis of substituent at C-4 (Table 2).

DISCUSSION

The sterol composition of buds is similar to that for anthers. For either buds or anthers, however, the free sterols are qualitatively different from the esterified sterols (Table 1). Esterified sterols accounted for 47 and 89% of total sterols of cotton buds and anthers, respectively. By comparison, the esterified sterol fraction constituted 75% of the total sterols of mixed pollen (10). The

TABLE 2

Results of Argentation Chromatography of Acetates of Combined Esterified Sterols from Cotton Buds and Anthers

Sterol acetates	Relative percentages of mass as sterol acetates[a]					
	Fraction 5 (7.0 mg)[b]	Fraction 6 (103.0 mg)[c]	Fraction 7 (29.0 mg)	Fraction 8 (53.2 mg)	Fraction 9 (9.2 mg)	Fraction 10 (10.0 mg)[d]
Pollinastanol	6.4	51.0				
31-Norcycloartanol	50.4	30.3				
Sitosterol			42.5			
24-Dehydropollinastanol			6.0	6.3	60.3	
31-Norcycloartenol			3.0	51.2	25.2	
Cycloartenol			41.3	41.9	6.2	
Desmosterol						24.1

[a]Determined by GLC.
[b]In this fraction, 21.9% of the mass is the acetate of dihydrophytol.
[c]In this fraction, 5 and 6.5% of the mass are the acetates of dihydrophytol and phytol, respectively.
[d]In this fraction, 20% of the mass is the acetate of geranylgeraniol plus the acetates of two other alcohols or sterols representing 28% of total mass.

esterified sterol fraction of cotton anthers contained the least amount of sitosterol. Our results thus indicate that the composition of the free sterols is mainly the end products of sterol biosynthesis in these two tissues, whereas the esterified sterol fraction is composed of mainly precursors and intermediates in the biosynthetic pathway to sitosterol or other sterol end products of the cotton plant.

Pollinastanol (1) was first isolated from a pollen (11), and 24-dehydropollinastanol (3), 31-norcycloartanol (2) and 31-norcycloartenol (4) have also been isolated from a mixture of pollens (10). Since an anther is part of a stamen that develops and contains pollen, it is not surprising that pollinastanol was found in cotton buds and anthers. The esterified sterol fraction of cotton buds and anthers contains all of the above-named sterols except 24-dehydropollinastanol and affords a convenient and accessible source of cycloartenol, an intermediate in the production of the common Δ^5-sterols in green plants (6). Both cycloartenol and 31-norcycloartanol have been isolated from pollen of *Taraxacum officinale* (12). Interestingly, 24-methylenecholesterol, which is often the principal sterol in pollen from a number of plant species (10,13-15), was present at only detectable levels in the free sterol fraction of cotton buds and anthers.

Sterols play an important role as structural components of membranes and as precursors to hormones or other compounds having physiological functions. All subcellular particles studied have been found to possess sterols (6). Although esters and glycosides do occur, usually the sterol is primarily in the free form (6). It has also been suggested that sterols in crop plants may have multiple nonmetabolic roles (16). In this study, the cotton buds contained about equal amounts of free and esterified sterols. On the other hand, cotton anthers contained a far greater percentage of their total sterols in the esterified form. The content of major free sterols increased in sorghum during the first 20 hr of germination, and the amount of free sterol of the mature leaf blades increased from day 7 to day 48, then decreased (16). The amount of sterol esters and glycosides was relatively low until flowering (16). Similar changes occur in sterol composition throughout the life cycle of soybean and squash (17). Thus the type of tissue, its age, the state of development, etc., can influence the qualitative and quantitative nature of sterols that occur during plant growth. To better understand the mechanism of biosynthesis and functions of sterols and the interrelationship between free and esterified sterols and sterol conjugates in the plant and animal kingdoms, then, the free and esterified sterols as well as sterol conjugates in the tissues should be qualitatively and quantitatively determined.

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Metabolism of Sterols of Varying Ring Unsaturation and Methylation by *Caenorhabditis elegans*

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The metabolism of three dietary 4,4-desmethylsterols and two 4α-methylsterols was investigated in the free-living nematode *Caenorhabditis elegans*. Dietary cholestanol was converted mostly to lathosterol. Dietary lathosterol, 7-dehydrocholesterol, 4α-methylcholest-7-enol and 4α-methylcholest-8(14)-enol each remained largely unchanged. An absolute requirement for a substantial quantity of 7-dehydrocholesterol in *C. elegans* did not exist. *C. elegans* was unable to remove a 4α-methyl group or introduce a double bond at C-5 and also demonstrated the lack of a Δ^7-reductase. Its nutritional sterol requirement was satisfied by cholestanol, lathosterol or 7-dehydrocholesterol; growth was comparable to that obtained previously in media containing Δ^5-sterols. However, the two 4α-methylsterols appeared to be unsatisfactory sterol nutrients. The possible physiological importance of 4α-methylsterols is discussed briefly.
Lipids 22, 84–87 (1987).

Nematodes are incapable of de novo sterol biosynthesis (1–4) and require exogenous sterol for normal growth and development (5–8). Metabolism of these exogenous sterols by nematodes is of current interest due to findings that several compounds with nematicidal activity also disrupt sterol metabolism in the free-living nematode *Caenorhabditis elegans* (9–11).

We recently discovered a unique metabolic process involving nuclear methylation of sterols at C-4 that occurs in three free-living nematodes, *C. elegans* (12), *Turbatrix aceti* and *Panagrellus redivivus* (13), but has not yet been reported in any other organism. Cultures of *C. elegans* propagated in media containing one of several dietary 4,4-desmethylsterols were found to produce 4-methylsterols, predominantly 4α-methylcholest-8(14)-enol with smaller amounts of lophenol (4α-methylcholest-7-enol) (9–14). Disruption of this 4-methylsterol pathway could provide for the design of selective nematicides against plant- and animal-parasitic species. 4-Methylsterols were not detected in cysts of *Heterodera zeae*, the corn cyst nematode (15), although the possibility of their occurrence in other life stages of this or other parasitic nematodes is under investigation.

Previously, with the exception of stigmastanol (14), we had only utilized Δ^5-sterols in the media, which were subsequently metabolized by *C. elegans* to produce mostly 7-dehydrocholesterol. Given the predominance of this $\Delta^{5,7}$-sterol, it became of interest to determine how non-Δ^5-sterols (Δ^0, Δ^7, $\Delta^{8(14)}$) would be metabolized and whether the presence of 7-dehydrocholesterol was an absolute requirement for growth and reproduction of *C. elegans*.

In the present study, sterols were isolated and identified from *C. elegans* propagated in media containing either cholestanol, 7-dehydrocholesterol, lathosterol, lophenol or 4α-methylcholest-8(14)-enol. Utilization of these dietary sterols, selected for their variation in ring unsaturation as well as their postulated intermediacy to 4α-methylcholest-8(14)-enol (11,14,15), provided further insight into sterol metabolism in *C. elegans.*

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MATERIALS AND METHODS

C. elegans was cultured axenically in semidefined aqueous media containing 25 mg/l sterol (10). Cholestanol (5α-cholestan-3β-ol) was obtained by catalytic reduction of cholesterol. 7-Dehydrocholesterol and lathosterol (5α-cholest-7-en-3β-ol) were commercial samples and were purified by column chromatography. Lophenol (4α-methylcholest-7-enol) and 4α-methylcholest-8(14)-enol were synthesized from lathosterol as described elsewhere (12). The purity of the dietary sterols (>98%) was assessed by capillary gas chromatography (GC).

Free and esterified sterols were isolated from lyophilized nematodes according to our earlier procedures (10, 14). Qualitative and quantitative analyses of sterols and their steryl acetate derivatives were performed by GC using both a glass column packed with 2.0% OV-17 liquid phase and a 15-m DB-1 capillary column. Final identifications were confirmed by capillary GC-mass spectrometry and UV spectroscopy (where applicable) and were in agreement with our previously described characteristics (14). Instrumental details have been supplied in an earlier paper (10).

RESULTS

C. elegans grew very well when propagated with either cholestanol, 7-dehydrocholesterol or lathosterol, as demonstrated by the population and dry weight values in Table 1, which are similar to those obtained previously when sitosterol was utilized as dietary sterol (10). Total lipid and sterol content values for these three desmethylsterol experiments (Table 1) did not differ appreciably from each other and are also similar to those obtained previously for a variety of dietary desmethylsterols including cholesterol, sitosterol and stigmasterol (9,14). Whether the dietary sterol was cholestanol, 7-dehydrocholesterol or lathosterol, most of the sterol from *C. elegans* was nonesterified; steryl esters comprised only 13–19% of the total sterol (Table 1).

By contrast, in the presence of either dietary 4-methylsterol, growth and reproduction of *C. elegans* were very poor. Dietary 4α-methylcholest-8(14)-enol resulted in population and dry weight values that were only 38% and 35%, respectively, of the average values yielded by the three dietary desmethylsterols. Reproduction was even poorer on lophenol, which yielded only 6% and 18% of the population and dry weight, respectively, obtained with desmethylsterol propagation. Although their total lipid contents were similar, the total sterol

TABLE 1

Effects of Dietary Sterols on Population Growth and Lipid and Sterol Content of *C. elegans*

Dietary sterol	Population (nematodes/ml)	Dry wt (g/l)	Total lipid[a] (% of dry wt)	Total sterol[b] (% of dry wt)	Esterified sterol (% of total sterol)
Cholestanol	72,600	1.47	18.0	0.12	19.1
7-Dehydrocholesterol	71,200	1.50	21.6	0.13	16.8
Lathosterol	84,000	1.80	21.4	0.13	12.9
Lophenol	4,600	0.28	23.9	0.23	—[c]
4α-Methylcholest-8(14)-enol	28,500	0.55	20.7	0.26	8.3

[a]Measured gravimetrically.
[b]Measured by GC quantitation.
[c]Not determined.

TABLE 2

Relative Percentages of Free Sterol (FS) and Steryl Ester (SE) Fractions from *C. elegans* Propagated with Various Dietary Sterols

	Dietary sterol							
	Stigmastanol[a]		Cholestanol		7-Dehydrocholesterol		Lathosterol	
Recovered sterol	FS	SE	FS	SE	FS	SE	FS	SE
Stigmastanol	14.2	30.1	—	—	—	—	—	—
Cholestanol	3.8	5.6	23.7	21.8	—	—	—	—
Cholesterol	—	—	—	—	0.3	1.2	—	—
7-Dehydrocholesterol	—	—	0.2	—	83.9	69.8	0.6	—
Cholesta-5,7,9(11)-trienol	—	—	—	—	7.4	6.4	0.1	—
Lathosterol	68.3	28.6	62.2	47.4	3.9	3.8	81.3	64.6
Cholest-8(14)-enol	3.7	6.4	1.7	3.7	0.1	0.5	3.1	5.1
4α-Methylcholest-7-enol	0.7	2.3	0.8	2.0	0.2	0.6	0.8	1.2
4α-Methylcholest-8(14)-enol	9.3	27.0	11.4	25.1	4.2	17.7	13.0	28.4
Others (unidentified)	—	—	—	—	—	—	1.1	0.7

[a]Stigmastanol data were reported previously (14).

content of 4-methylsterol-propagated cultures was greater than that of desmethylsterol-propagated cultures, possibly due to a greater uptake of the 4-methylsterols by *C. elegans*.

The compositions of free sterol and steryl ester fractions from *C. elegans* propagated with cholestanol, 7-dehydrocholesterol, lathosterol or stigmastanol (from a previous study [14]) are presented in Table 2. *C. elegans* propagated with cholestanol contained, excluding dietary sterol, predominantly lathosterol with lesser amounts of 4α-methylcholest-8(14)-enol and cholest-8(14)-enol, as it did similarly when propagated with another stanol, stigmastanol. The major sterol of *C. elegans* propagated with 7-dehydrocholesterol was dietary sterol with lesser amounts of cholesta-5,7,9(11)-trienol, 4α-methylcholest-8(14)-enol and lathosterol. Lathosterol-fed *C. elegans* contained mostly dietary sterol and smaller quantities of 4α-methylcholest-8(14)-enol and cholest-8(14)-enol. The free sterol compositions were generally similar to the esterified sterol profiles with the exception of 4α-methylcholest-8(14)-enol, which, in all cases, comprised a fairly large percentage of the ester fraction. This phenomenon has been observed in all of our previous sterol analyses.

Table 3 lists the total sterol compositions of *C. elegans* propagated with each of the two 4-methylsterols. Lophenol-propagated cultures contained predominantly dietary sterol and a small but significant percentage of 4α-methylcholest-8(14)-enol. *C. elegans* fed 4α-methylcholest-8(14)-enol contained almost exclusively dietary sterol.

DISCUSSION

In our previous studies (9,11,14), the nutritional requirement for sterols in *C. elegans* was satisfied by a number of Δ^5-sterols, which were subsequently metabolized to produce predominantly 7-dehydrocholesterol, a $\Delta^{5,7}$-sterol. In the present study, cholestanol and a Δ^7-sterol (lathosterol) each performed as well as the Δ^5-sterols in satisfying the nutritional requirement. Dietary cholestanol and stigmastanol were converted mostly to lathosterol, while dietary lathosterol remained largely unchanged. In *C. elegans* fed either cholestanol or lathosterol, we detected a very small quantity of 7-dehydrocholesterol, which was probably of exogenous origin rather than a metabolite of the supplemented cholestanol and lathosterol. Therefore, even though 7-dehydrocholesterol is the major metabolite in

TABLE 3

Relative Percentages of Total Sterols from *C. elegans* Propagated with Dietary 4-Methylsterols

Recovered sterol	Dietary sterol	
	4α-Methylcholest-7-enol	4α-Methylcholest-8(14)-enol[a]
Cholesterol	0.6	0.6
Cholest-8(14)-enol	0.2	0.4
Lathosterol	0.8	0.7
Campesterol	—	1.1
Sitosterol	0.6	0.7
4α-Methylcholest-7-enol	87.3	1.6
4α-Methylcholest-8(14)-enol	10.5	94.9

[a]Contained less than 1% 4α-methylcholest-7-enol by GC.

C. elegans fed Δ^5-sterols, *C. elegans* has no absolute requirement for a substantial quantity of 7-dehydrocholesterol. Its structural requirement for sterol can also be fulfilled by lathosterol. Much of the dietary 7-dehydrocholesterol remained unchanged. A relatively small amount of cholesterol was present in 7-dehydrocholesterol-fed *C. elegans,* whose origin may have been as a media contaminant rather than as a metabolite of the supplemented 7-dehydrocholesterol. It is most probable that *C. elegans* lacks both a C-5 dehydrogenase and a Δ^7-reductase. Some of the dietary lophenol was converted to its $\Delta^{8(14)}$-analog; most of the dietary 4α-methylcholest-8(14)-enol (or possibly all of it) was not metabolized. In each case, we found only small percentages of desmethylsterols, which probably were exogenous media contaminants not completely extracted in our media preparation. Therefore, *C. elegans* has little or no ability to demethylate either of these two 4α-methylsterols.

While a number of 4,4-desmethylsterols have been shown to fulfill the nutritional requirement of *C. elegans,* neither of the two 4α-methylsterols tested here appeared satisfactory. Failure could possibly be due to the inability of a 4α-methylsterol to function as a bulk membrane component in *C. elegans,* and perhaps this nematode biosynthesizes 4α-methylsterols for a more specific function such as a hormone, a pheromone or a precursor to these, as previously suggested (9). Alternatively, if the 4α-methylsterol could indeed fulfill the bulk membrane role, normal growth and reproduction might not occur if an insufficient quantity of 4-desmethylsterols were available to fill some critical role requiring a structure-specific sterol (e.g., 7-dehydrocholesterol or lathosterol). Evidence for such multiplicity of sterol functions has been demonstrated by sterol "sparing" effects in insects (16) and "sparking" effects in fungi (17-19). Failure to make indirect use of 4α-methylsterols stems from the inability of *C. elegans* to convert them to presumably functional 4-desmethylsterols.

The compositions of recovered sterols from *C. elegans* propagated with the various dietary sterols (Tables 2 and 3) substantiate our previously hypothesized metabolic pathways (20). Figure 1 summarizes the nuclear modifications leading toward the production of 4α-methylcholest-8(14)-enol in *C. elegans.*

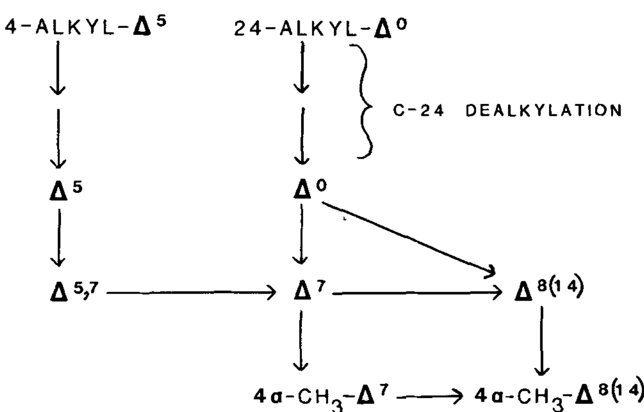

FIG. 1. Metabolic pathways to 4α-methylsterols in *C. elegans.*

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The Effects of Fat-Free, Saturated and Polyunsaturated Fat Diets on Rat Liver and Plasma Lipids

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The liver and plasma lipids and fatty acid composition of rats fed synthetic diets of differing fat type and content were studied. All animals were starved for 48 hr and then refed a high carbohydrate, fat-free diet for 48 hr. They were then divided into three groups and fed for an additional 48 hrs the following: group 1, the fat-free diet; group 2, a diet containing 44% of calories from corn oil; and group 3, a diet containing 44% calories from completely hydrogenated soybean oil. The total lipid concentration of the liver in the animals on the fat-free diet was elevated at 72 and 96 hr. The addition of either saturated or unsaturated fat in the diet at 48 hr prevented this accumulation. The total phospholipid and cholesterol concentrations of the liver were relatively uninfluenced by any diet in this study. Plasma total fatty acid concentration was elevated at 72 hr in the animals on a fat-free diet compared to those fed the stock diet, starved for 48 hr or fed the fat-containing diets. By 96 hr, however, plasma fatty acid concentrations in all groups were similar to those in animals fed only the stock diet. The release of de novo synthesized fatty acids into plasma from the liver was strongly inhibited by dietary fat, either saturated or polyunsaturated. With the fat-free diet there was a significant increase in the saturated and monounsaturated fatty acids in both liver and plasma. The addition of corn oil to the diet facilitated a reversion of the fatty acid composition in liver and plasma to that found in the animals fed the stock diet ad libitum, but saturated fat did not. No effect of diet on the fatty acid composition of the red cells was observed during the course of this study. Exogenous saturated fatty acids, although similar chemically to the fatty acids synthesized by the liver, may have physiological actions that differ from endogenously synthesized fat.
Lipids 22, **88–94 (1987).**

Dietary fat has profound effects on endogenous fat metabolism, and these effects differ depending upon the type of fat consumed (1–3). Fat-free diets in the rat stimulate liver fatty acid synthesis (2,4–6), while polyunsaturated fat in the diet strongly inhibits endogenous fatty acid synthesis (7–10). The effect of saturated fat in the diet on the suppression of endogenous fatty acid synthesis is still controversial (11–13).

This research was undertaken to elucidate how short-term changes in dietary fats influence endogenous fatty acid synthesis in the rat in vivo because of the marked effects observed in cultured rat hepatocytes (14,15). It was also of interest to determine how dietary fat composition affects the content and composition of tissue lipids. This report presents the effects of short-term fat feeding and type of dietary fat on liver and plasma lipid levels and the time course of changes in the fatty acid composition of the liver and plasma. In addition, release of de novo synthesized fatty acids into the plasma as a function of time and type of diet was determined.

MATERIALS AND METHODS

Materials, animals and diets. Male Sprague-Dawley rats, 100–150 g, were purchased from Bantin and Kingman (Fremont, California). They were fed a stock laboratory diet from Ralston-Purina (Richmond, Indiana) until they had grown to 400–500 g. At 120–140 days of age, the animals were segregated into three groups of 12 to 22 rats each, starved for 48 hr and refed a fat-free, high carbohydrate (FF) diet for 48 hr. After that, one group was continued on the FF diet for another 48 hr. A second group was placed on a synthetic diet in which 44% of the calories was supplied by corn oil (CO). The remaining group was fed a diet in which 44% of the calories was supplied by completely hydrogenated soybean oil (HSO) (a gift of Durkee Foods, Cleveland, Ohio) for 48 hr. The rats fed the stock diet were not starved and refed. The composition of the three diets is given in Table 1; that of the dietary fats is listed in Table 2. The vitamin and mineral mix (AIN-76) added to the synthetic diets was from Teklad Diets (Madison, Wisconsin). Animals had access to the diets ad libitum except initially, when all animals were given ca. 3 ml of the diet by stomach tube at the start of refeeding both the fat-free and fat-containing diets. All animals consumed approximately the same amount of calories regardless of diet group and appeared to find the diets quite palatable.

The animals were killed at 0, 3, 9, 24, 48, 51, 57, 72 and 96 hr after refeeding was begun. All animals received intraperitoneally ^3H_2O one hour before being killed (0.5 mCi/100 g body weight).

The liver and other tissues were removed and either extracted immediately or frozen at −20 C until they were extracted. Blood was drawn into heparinized syringes from the inferior vena cava. Red cells and plasma were separated by centrifugation at 3600 × g for 15 min. The red cells were washed (4×) with isotonic phosphate-buffered saline.

TABLE 1

Composition of Diets

Component	Wt % Fat-free diet	Corn oil diet	Hydrogenated soybean oil diet
Sucrose	70.5	43.0	43.0
Glycerol	5.0	—	—
Egg albumin	20.0	27.9	27.9
Mineral mix (AIN-76)	3.5	3.3	3.3
Vitamin mix (Teklad #40060)	1.0	1.0	1.0
Fat	—	24.8	24.8

Caloric distribution for the fat-free diet is the same as the weight %; that for fat-containing diets is 34% sucrose, 22% albumin and 44% fat.

TABLE 2

Fatty Acid Composition of Experimental Diets (wt % of Total Fatty Acids)

Fatty acid[a]	Corn oil diet	Hydrogenated soybean oil diet
14:0	0.12	0.12
16:0	11.23	10.72
16:1(n-7)	0.10	—
17:0	0.14	0.25
18:0	2.06	86.37
18:1(n-9)	25.55	0.32
18:1(n-7)	1.04	—
18:2(n-6)	55.02	—
18:3(n-3)	0.42	—
20:0	0.46	0.55
20:1(n-9)	0.25	—
20:4(n-6)	0.09	—
22:0	0.16	—
24:0	—	0.11
Sum of trace components	2.96	1.21

[a]Fatty acids are designated by the carbon chain length, number of double bonds and the position of the first double bond from the methylene end of the molecule.

All organic solvents were obtained from Burdick and Jackson (Muskegon, Michigan). Purified fatty acid methyl ester (FAME) reference standards were purchased from Nu-Chek-Prep (Elysian, Minnesota).

Lipid extraction. The lipid extractions were performed using $CHCl_3$-MeOH (2:1, v/v) by the procedures described previously (16,17) after lyophilization to remove all free and tritiated water. Hydroquinone was added to all samples. The purified total lipid extracts (TLE) were either prepared for transmethylation immediately or stored under nitrogen at −20 C until further processing.

Phospholipid analysis. The total phospholipid content (in mg) of the TLE was estimated from the mg of inorganic phosphate × 25 (18).

Cholesterol determination. Cholesterol was determined by a gas liquid chromatography (GLC) method. An aliquot of the TLE was dissolved in $CHCl_3$ to a concentration appropriate for direct injection onto a fused silica capillary column. The column was coated with a cross-linked methyl silicone polymer (Ultra-1 column, 25 m × 0.25 mm I.D., obtained from the Hewlett-Packard Corp., Palo Alto, California). The gas chromatograph was a Hewlett-Packard 5880A equipped with a Hewlett-Packard electronic integrator and data processor. Conditions were as follows: carrier gas, He at 14.5 psi; injection port temperature, 250 C; oven temperature, 280 C; FID temperature, 300 C; split ratio 50:1.

Under these operating conditions, cholesterol eluted from the column as a clean peak at 12.5 min. There was no interference from the other components in the TLE. Various peaks attributable to triglycerides, fatty acids and decomposition products of phospholipids appeared on the chromatogram, but always at retention times of 9 min or less under these chromatographic conditions.

Cholesterol was quantitated using peak areas compared to area obtained from a high purity cholesterol reference standard from Sigma Biochemicals (St. Louis, Missouri). The calibration was linear from 50 ng to 20 mg per μl of cholesterol. The cholesterol peaks showed some tailing at the higher concentrations, but this did not affect quantitation. Integration of peak areas, use of correction factors and calculation of the absolute amounts of cholesterol in the sample were performed automatically by the data station after calibration.

Tritium-labeled liver cholesterol samples were prepared for liquid scintillation counting by separation on thin layer plates (16), using pre-coated silica-gel plates obtained from E-M Science (Cherry Hill, New Jersey). The developing solvent was hexane/diethyl ether/acetic acid, (85:15:2, v/v/v). The cholesterol spot was located with iodine vapor and scraped into a scintillation vial without eluting the cholesterol from the absorbent. The presence of the adsorbent in the scintillation vial did not significantly interfere with the tritium counting.

Transmethylation. The transmethylations to obtain FAME were carried out using methanolic-HCl (7%, w/w) (14). FAME were diluted in hexane to an appropriate concentration and stored at −20 C until analyzed by GLC or by liquid scintillation counting of ^3H in their fatty acid moiety.

GLC of FAME. The FAME samples were analyzed as described previously (14) on fused silica capillary columns coated with SP-2340 (Supelco, Bellefonte, Pennsylvania). Samples were chromatographed on a Perkin-Elmer Sigma 2000 coupled to a P-E 7500 computer loaded with a Chrom 3 data analysis program.

The quantitative accuracy of the GLC procedures was evaluated by using either purified single FAME or reference mixtures selected to cover the range of FAME present in the experimental samples. Accuracy of the analysis was estimated to be within 5% for the major components (greater than 10% of the total FAME in the sample) and within 10% for the minor components in the samples.

The cholesterol extracted into the hexane washes of the transmethylation procedure was not separated from the FAME prior to injection of the samples in the chromatograph. However, cholesterol is largely decomposed during acidic transmethylation (19), and free cholesterol does not elute as a discrete peak from a SP-2340 column under the conditions in this work (14).

The composition (wt %) of the FAME in the rat samples were derived from the area percentages on the chromatograms (14). A chromatogram had between 40 and 80 discrete FAME peaks. Most minor peaks contributed less than 0.1% to the total area individually and were not identified with confidence. The major identified components usually comprised 95–97% of the total FAME present in the sample. For convenience, only selected major fatty acids are listed in the tables. The residue is collectively presented as "sum of trace components" and varies from approximately 4 to 12% of the total fatty acid present in the samples. Both identified and unidentified components are grouped in this category.

In some of the tables and figures, data are presented in terms of mg lipid/mg cellular protein because this allows the amounts to be compared in absolute terms rather than by the normalized values given by the usual wt % calculations. Proteins were analyzed by the method of Lowry et al. (20). The data were analyzed statistically using the two-tailed Student's t-test.

TABLE 3

Effect of Dietary Treatments on the Wet Weight, Lipid and Protein Content of Rat Liver[a]

	Stock diet (n = 5)	Starved 48 Hr (n = 4)	48 Hr, refed FF diet (n = 5)	96 Hr, refed FF diet (n = 4)	48 Hr, refed FF diet: 48 Hr CO diet (n = 5)	48 Hr, refed FF diet: 48 Hr HSO diet (n = 6)
Wet weight of liver (g)	16.35 ±0.51	12.04* ±1.60	21.83* ±1.36	25.13* ±2.59	17.55 ±1.43	16.88 ±2.55
Total lipid (% of wet wt)	5.75 ±1.35	6.90 ±1.68	6.73 ±1.03	15.82* ±4.35	8.41* ±0.83	7.78* ±1.36
mg Protein ÷ g wet wt liver	147.0 ±10.9	164.4* ±8.0	136.4 ±22.4	123.8* ±5.8	142.9 ±12.0	142.9 ±7.1

[a]Abbreviations: FF, fat-free; CO, corn oil; HSO, hydrogenated soybean oil.
*Significantly different from the stock diet at $P < 0.05$.

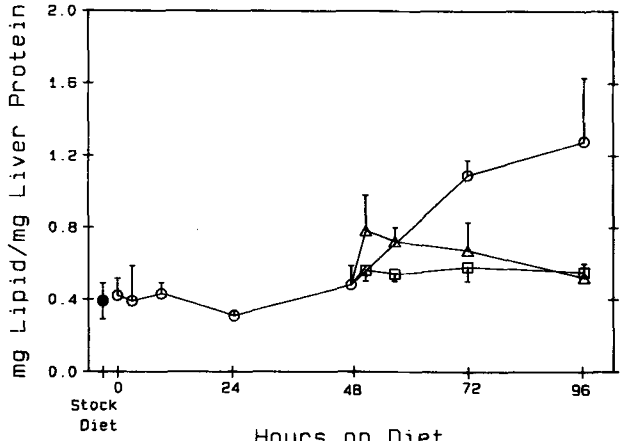

FIG. 1. The total lipid concentration (mg total lipid/mg liver protein) of liver for rats fed the various diets. ○, Fat-free, high carbohydrate diet; □, corn oil diet; △, hydrogenated soybean diet; ●, stock diet. Data shown are means ±S.D. for 3 to 6 animals. Only the 72- and 96-hr points for the animals on the fat-free diet are significantly different from the other points ($p < 0.05$ for the 72-hr point and $p < 0.01$ for the 96-hr point). Dietary protocols are given in the text.

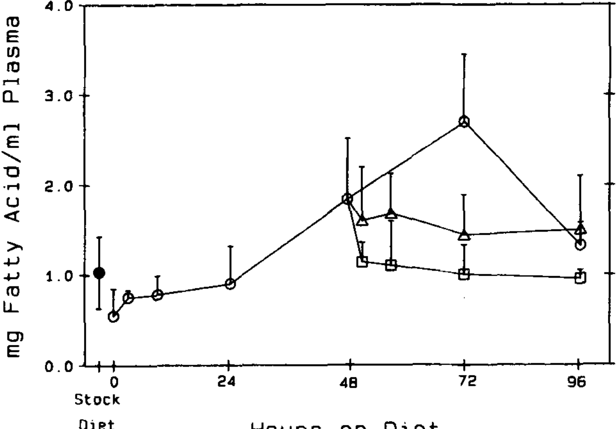

FIG. 2. Plasma total fatty acid concentration from rats on the three dietary treatments. ○, Fat-free, high carbohydrate diet; □, corn oil diet; △, hydrogenated soybean diet; ●, stock diet. Only the 72-hr data point for the animals on the fat-free diet is significantly different from the other data points ($p < 0.05$).

RESULTS

Table 3 lists average weights, percent lipid and protein per g of liver for the experimental animals on the various diets fed in this study. The wet weight of the liver decreased markedly after 48 hr of starvation and increased rapidly on the fat-free diet. The addition of either saturated or polyunsaturated fat to the diet caused a reversal of this trend and a return to the average wet weight found in the stock-fed animals. The percent lipid in the livers of these animals increased on the fat-free diet but decreased when either fat was added. The absolute amount of protein per cell varies little in these short-term feeding studies (14,15); hence, it is advantageous to present changes in the lipid content of the liver in terms of mg lipid/mg liver protein, as this value is relatively uninfluenced by changes in liver hydration and glycogen content that tend to confound percent weight calculations.

Figure 1 shows the time course of changes in the total lipid concentration of rat liver in mg lipid/mg liver protein as a function of the three dietary treatments. The liver lipids of animals on the fat-free diet show a significant elevation at 72 and 96 hr. The addition of fat to the diet after 48 hr of refeeding a FF diet prevented this increase in liver lipid concentration. This reduction was noted within 3 hr with the corn oil diet and within 24 hr with the saturated fat diet. By 48 hr on either fat-containing diet, the liver lipid level had equalized at about 8% of the liver wet weight, considerably below that found in the animals fed the FF diet.

Figure 2 presents the total fatty acid concentration of the plasma in the three groups. By 96 hr the plasma fatty acid content had reached a similar level (in mg fatty acid/ml plasma) in all three groups of animals, regardless of diet, in contrast to the liver lipid concentration, which demonstrated a marked difference between the fat-fed and fat-free diet groups at 96 hr. The animals on the fat-free diet did have elevated plasma lipid levels at 72 hr, while the plasma lipids in the fat-fed animals did not show a significant rise at this time. The slight elevation of the

plasma fatty acid in the animals fed unsaturated fat was not statistically significant.

Figure 3 displays the phospholipid concentration of the livers in the animals on the different diets. The ordinate in this figure is given in terms of mg phospholipid/mg of liver protein. Once again, the ordinate scale was chosen because this is approximately equivalent to a per-cell measurement unaffected by hydration or liver glycogen content (15). Expressed in these units, the phospholipid level of the liver changed only slightly during the starvation and refeeding periods and was relatively uninfluenced by the fat type or level in the diet.

Figure 4 shows the cholesterol concentration of the livers of the animals on the various diets during the feeding period. Similar to the phospholipids of the liver, cholesterol was not significantly influenced by the diets.

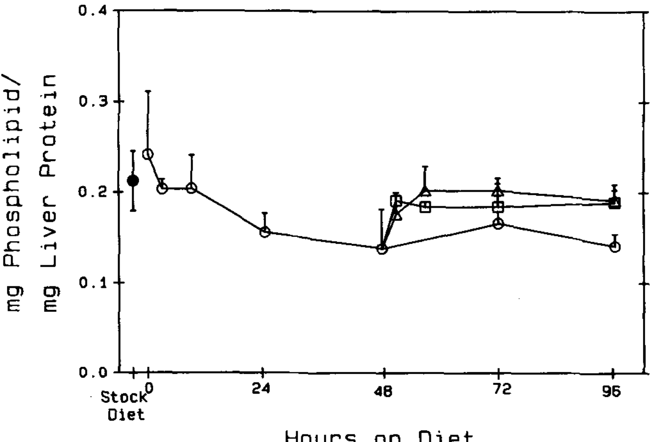

FIG. 3. Total phospholipid concentration of the livers from rats on the three dietary treatments. ○, Fat-free, high carbohydrate diet; □, corn oil diet; △, hydrogenated soybean diet; ●, stock diet. While a slight downward trend in the phospholipid content of the liver in animals on the fat-free diet was noted, no statistically significant differences were detected among the three dietary treatments.

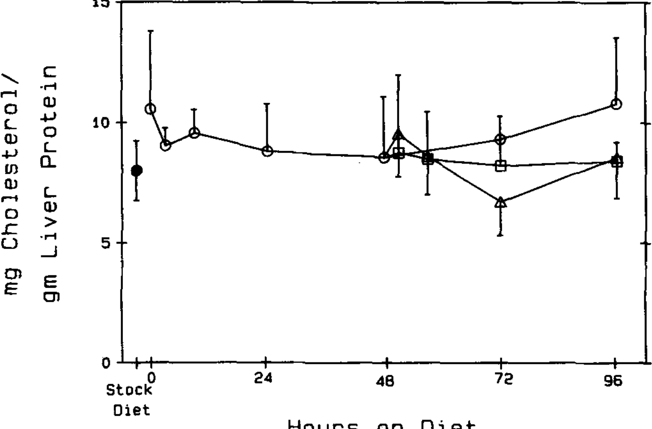

FIG. 4. Total cholesterol content of the liver in rats on the three dietary treatments. ○, Fat-free, high carbohydrate diet; □, corn oil diet; △, hydrogenated soybean diet; ●, stock diet. No significant differences in the liver cholesterol concentration were detected among the three dietary treatments.

In addition, when the cholesterol fraction of the liver was isolated and counted, no significant activity could be detected, indicating that very little de novo synthesis of cholesterol had occurred during the time the animals were receiving the experimental diets devoid of exogenous cholesterol.

Table 4 lists the fatty acid composition of the livers of the animals in the three dietary groups at 0, 24, 48, 72 and 96 hr. The fat-free and the saturated-fat diet groups had very similar fatty acid compositions in their livers during this study, markedly different from that observed in stock-fed animals. When corn oil was added to the diet, there was an immediate and marked change in the fatty acid composition of the liver—within 48 hr it reverted to that observed in the stock-fed animals.

Rat liver appears to show a marked preference for polyunsaturated fatty acids such as linoleic acid. In animals fed corn oil for 24 hr, the liver linoleic acid content increased from 2% to 15% and was approximately equal to that of the animals fed stock diet ad libitum. This would indicate a replacement of monounsaturated and saturated fatty acids by linoleic acid in the liver. Liver fatty acid composition was similar in animals fed saturated-fat and fat-free diets. Stearic acid accounted for approximately 40% of calories in the saturated fat diet, but little accumulated in liver.

Table 5 presents the fatty acid composition of plasma in the different dietary groups at 0, 24, 48, 72 and 96 hr. Ingested linoleic acid caused a rapid rise (within 3 hr) in the animals' plasma linoleic acid levels; stearic acid ingestion did not cause a marked rise in the plasma stearic acid levels. No major differences were observed between the fatty acid compositions of the plasma of the fat-free and the saturated-fat diet groups. After 3 hr on the 44% saturated-fat diet, the plasma stearic acid content rose only 3%; it increased by 6% after 48 hr. The palmitoleic acid content of the plasma was the most markedly affected by the corn oil diet; it decreased from 8.2% at the start of corn oil feeding to 0.8% after 48 hr. The levels of the other major fatty acids of the plasma were not as affected by the corn oil diet as the level of palmitoleic acid.

All percentages for the major fatty acids in liver and plasma for the various diet groups listed in Tables 4 and 5 were significantly different from those in the liver and plasma of animals in the group starved for 48 hr.

The fatty acid composition of the red blood cells in the animals in the three diet groups showed no significant changes during the course of this study (data not shown).

Figure 5 displays the activity (in DPM) of tritium-labeled fatty acids found in plasma of animals in the various dietary groups. The maximum release was achieved at 48 hr on the fat-free diet, and it decreased markedly by 96 hr. However, this release of de novo synthesized fatty acids into the blood was strongly inhibited by dietary fat. The amount of activity in the fatty acids in plasma after 3 hr on either the saturated fat or polyunsaturated fat diets was equally diminished. The apparent difference between the shape of the curve of the saturated and unsaturated diet groups is not statistically significant.

DISCUSSION

This report extends earlier studies on the effect of dietary fat on liver and plasma lipid and their fatty acid

TABLE 4

Fatty Acid Composition of Rat Liver as Affected by Dietary Regimen (Wt % of Total Liver Fatty Acids)

		0 Hr	24 Hr	48 Hr	72 Hr			96 Hr		
Fatty acids[a]	Stock diet, fed ad libitum (n = 5)	Starved 48 hr (n = 4)	Fat-free diet (n = 5)	Fat-free diet (n = 5)	Fat-free diet (n = 4)	24 Hr saturated fat diet (n = 4)	24 Hr corn oil diet (n = 5)	Fat-free diet (n = 4)	48 Hr saturated fat diet (n = 4)	48 Hr corn oil diet (n = 5)
14:0	0.33 ± 0.09	0.24 ± 0.06	0.69 ± 0.12	1.46 ± 0.12	1.78 ± 0.27	1.69 ± 0.15	1.12 ± 0.10	1.99 ± 0.28	1.69 ± 0.15	0.70 ± 0.33
16:0	14.13 ± 1.05	18.64 ± 2.56	25.87 ± 1.44	28.96 ± 1.31	33.08 ± 0.63	31.08 ± 1.54	23.98 ± 1.22	36.76 ± 1.73	31.08 ± 1.54	21.41 ± 3.51
16:1(n-9)	0.30 ± 0.04	0.18 ± 0.05	0.34 ± 0.03	0.58 ± 0.07	0.71 ± 0.06	0.71 ± 0.09	0.50 ± 0.07	0.99 ± 0.06	0.71 ± 0.09	0.35 ± 0.17
16:1(n-7)	1.24 ± 0.33	0.82 ± 0.33	7.47 ± 1.94	13.67 ± 0.86	10.84 ± 1.59	10.84 ± 1.17	6.44 ± 1.65	12.26 ± 1.25	10.84 ± 1.17	2.09 ± 1.09
18:0	14.03 ± 2.37	16.61 ± 3.03	10.41 ± 1.49	5.46 ± 0.53	6.97 ± 0.58	6.97 ± 1.38	8.39 ± 0.44	4.97 ± 0.81	6.97 ± 1.38	11.70 ± 4.14
18:1(n-9)	11.92 ± 1.98	9.09 ± 2.32	16.84 ± 1.72	27.38 ± 2.97	28.17 ± 1.46	28.52 ± 2.22	23.19 ± 1.42	29.21 ± 0.67	28.52 ± 2.22	16.79 ± 4.90
18:1(n-7)	3.80 ± 0.92	1.87 ± 0.10	4.67 ± 1.04	6.71 ± 0.68	6.38 ± 1.46	6.27 ± 0.07	4.35 ± 0.43	5.53 ± 0.40	6.27 ± 0.71	2.64 ± 0.40
18:2(n-6)	16.82 ± 1.09	17.45 ± 1.94	9.52 ± 1.34	2.15 ± 0.04	1.11 ± 0.03	1.81 ± 0.71	14.81 ± 1.88	0.81 ± 0.20	1.81 ± 0.71	18.99 ± 2.56
22:1(n-11) + 20:4(n-6)	13.52 ± 2.38	18.03 ± 4.59	11.88 ± 1.56	4.28 ± 0.84	2.98 ± 0.48	4.34 ± 1.38	7.14 ± 2.21	2.11 ± 0.64	4.34 ± 1.38	12.25 ± 4.96
20:5(n-3)	1.00 ± 0.17	1.10 ± 0.39	0.59 ± 0.03	0.21 ± 0.07	0.26 ± 0.05	0.16 ± 0.05	0.10 ± 0.01	0.20 ± 0.07	0.16 ± 0.05	0.09 ± 0.06
22:5(n-3)	2.16 ± 0.48	1.35 ± 0.11	0.59 ± 0.11	0.18 ± 0.08	0.12 ± 0.04	0.12 ± 0.07	0.19 ± 0.07	0.10 ± 0.01	0.12 ± 0.07	0.25 ± 0.21
22:6(n-3)	7.40 ± 0.48	8.30 ± 0.49	5.84 ± 0.53	2.24 ± 0.55	1.57 ± 0.27	2.26 ± 0.76	2.60 ± 0.81	1.28 ± 0.39	2.26 ± 0.76	3.38 ± 2.44
Sum of trace components	8.40 ± 0.48	5.66 ± 0.55	5.29 ± 0.64	6.72 ± 2.84	4.37 ± 0.37	5.23 ± 0.98	7.19 ± 0.35	3.79 ± 0.50	5.23 ± 0.93	8.76 ± 0.55

[a]Fatty acids designated by chain length, number of double bonds and the position of the first double bond from the methylene end of the molecule.

TABLE 5

Fatty Acid Composition of Rat Plasma as Affected by Dietary Regimen (Wt % of Total Plasma Fatty Acids)

		0 Hr	24 Hr	48 Hr	72 Hr			96 Hr		
Fatty acids[a]	Stock diet, fed ad libitum (n = 5)	Starved 48 hr (n = 3)	Fat-free diet (n = 5)	Fat-free diet (n = 5)	Fat-free diet (n = 4)	24 Hr saturated fat diet (n = 4)	24 Hr corn oil diet (n = 4)	Fat-free diet (n = 4)	48 Hr saturated fat diet (n = 5)	48 Hr corn oil diet (n = 5)
14:0	0.55 ± 0.13	0.24 ± 0.06	0.85 ± 0.17	1.54 ± 0.10	1.45 ± 0.18	1.02 ± 0.07	0.30 ± 0.07	0.75 ± 0.21	0.98 ± 0.09	0.22 ± 0.06
16:0	19.53 ± 1.61	15.18 ± 0.64	25.93 ± 1.99	27.36 ± 0.77	27.40 ± 1.29	21.35 ± 1.64	16.36 ± 0.84	23.93 ± 2.30	22.93 ± 2.66	15.73 ± 2.18
16:1(n-9)	0.30 ± 0.63	0.19 ± 0.04	0.41 ± 0.04	0.64 ± 0.03	0.86 ± 0.14	0.77 ± 0.18	0.27 ± 0.04	1.09 ± 0.03	1.02 ± 0.25	0.23 ± 0.03
16:1(n-7)	1.64 ± 0.27	0.78 ± 0.09	9.52 ± 1.99	13.33 ± 1.13	11.34 ± 1.79	7.09 ± 1.27	1.44 ± 0.20	8.73 ± 1.40	6.97 ± 2.00	0.77 ± 0.19
18:0	9.80 ± 1.20	12.20 ± 0.62	5.79 ± 0.93	5.21 ± 0.70	7.82 ± 1.31	11.88 ± 0.09	10.45 ± 0.62	10.87 ± 1.96	11.04 ± 0.44	10.89 ± 2.33
18:1(n-9)	12.95 ± 1.40	6.12 ± 0.96	21.66 ± 3.08	27.65 ± 3.18	24.62 ± 1.55	22.11 ± 2.28	16.55 ± 0.58	23.92 ± 3.93	21.31 ± 1.38	15.46 ± 3.82
18:1(n-7)	2.95 ± 0.52	1.52 ± 0.25	5.68 ± 1.08	8.25 ± 0.57	7.19 ± 0.85	6.23 ± 0.44	2.42 ± 0.28	6.08 ± 0.44	6.18 ± 0.50	1.67 ± 0.34
18:2(n-6)	22.41 ± 0.88	12.50 ± 1.40	7.79 ± 1.51	2.11 ± 0.61	2.21 ± 0.29	3.94 ± 0.92	31.28 ± 2.04	2.46 ± 0.34	3.68 ± 0.54	33.38 ± 6.43
22:1(n-11) + 22:4(n-6)	13.39 ± 3.11	35.70 ± 3.12	10.40 ± 2.76	4.62 ± 1.00	6.23 ± 1.29	11.19 ±3.20	10.61 ± 0.83	8.42 ± 1.99	11.29 ± 0.88	11.87 ± 4.34
20:5(n-3)	1.63 ± 0.25	1.00 ± 0.13	0.53 ± 0.10	0.20 ± 0.10	0.30 ± 0.07	0.62 ± 0.18	0.14 ± 0.07	0.44 ± 0.07	0.48 ± 0.36	0.06 ± 0.01
22:5(n-3)	0.73 ± 0.11	0.69 ± 0.03	0.36 ± 0.11	0.17 ± 0.06	0.15 ± 0.04	0.20 ± 0.06	0.34 ± 0.26	0.16 ± 0.13	0.28 ± 0.12	0.24 ± 0.15
22:6(n-3)	3.55 ± 0.59	4.66 ± 0.88	2.68 ± 0.42	1.46 ± 0.39	1.77 ± 0.28	2.58 ± 0.55	1.59 ± 0.64	2.39 ± 0.31	2.76 ± 0.80	1.41 ± 1.06
Sum of trace components	9.78 ± 0.90	8.98 ± 1.01	8.18 ± 1.56	7.66 ± 0.58	8.46 ± 1.08	10.83 ± 1.50	8.25 ± 0.99	11.36 ± 3.07	11.69 ± 1.57	8.05 ± 1.75

[a]Fatty acids designated by chain length, number of double bonds and the position of the first double bond from the methylene end of the molecule.

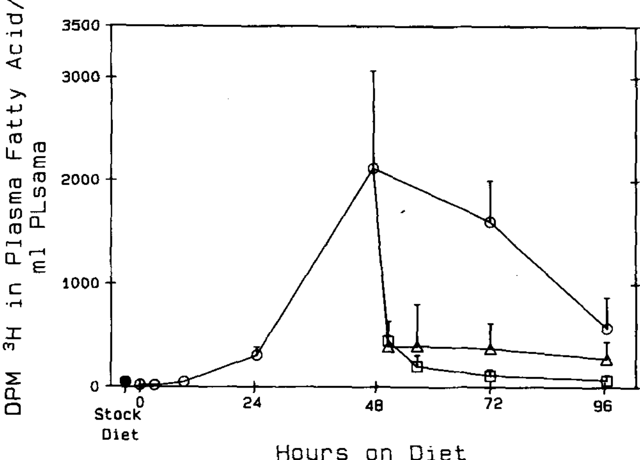

FIG. 5. Activity of ^3H of plasma fatty acid in DPM/ml plasma for rats on the three dietary treatments. ○, Fat-free, high carbohydrate diet; □, corn oil diet; △, hydrogenated soybean diet; ●, stock diet. The 48- and 72-hr points for the animals on the fat-free diet are significantly different from the other dietary groups (48 hr, $p < 0.05$; 72 hr, $p < 0.01$). The 96-hr point for the fat-free diet group did not reach significance at the 0.05 level. Refer to text for details of the experimental conditions.

compositions. It differs from previous studies in that the time course of changes during the first 96 hr of feeding are presented. These results indicate that refeeding a fat-free, high carbohydrate diet to animals starved for 48 hr causes an increase in the lipid concentration of the liver, as previously reported by ourselves (16) and others (6,11,21-23), which is almost exclusively in the triglyceride fraction of the liver lipids. Nace and Szepesi (6) reported that animals starved for 48 hr and refed fat-containing diets did not exhibit the increase in liver lipids observed in animals refed a FF diet after three days of refeeding. Those authors did not, however, refeed a fat-containing diet to animals that had first been refed a FF diet. The work reported here demonstrates that even after induction of active lipogenesis by a FF diet, addition of fat to the diet rapidly halts the increase in liver lipid accumulation.

In our work, the nutrient densities of the fat-free and fat-containing diets were quite different with respect to the vitamin and mineral content. However, it is unlikely that during the 96 hr of refeeding these differences would markedly influence any of the metabolic processes examined. Also, the digestibility of corn oil and hydrogenated soybean oil is very different. For this reason, diets with a high level of fat were deliberately chosen to insure that an adequate amount of the saturated fat would be absorbed and able to influence liver fatty acid metabolism. It is possible that some of the differences in fatty acid composition found among the tissues in animals consuming the two different diets are the result of this digestibility difference in saturated and unsaturated fatty acids. Nevertheless, our data suggest that dietary saturated and unsaturated fatty acids have similar influence on fat metabolism when consumed in appropriate amounts.

It should also be noted that the increases in wet weight and lipid content of the liver in our animals were somewhat greater than those reported by other investigators (6,12, 22). The animals used in this study were older and larger than rats of 150 to 200 g frequently used by others, which may well account for the differences.

The hydrogenated soybean oil diet contained mainly palmitic (11%) and stearic (86%) acids. These two compounds are also the primary products of fatty acid synthesis in rat liver (24,25) in animals on a fat-free diet, so there is no obvious reason why their addition to the diet should reduce liver lipid levels. Product feedback inhibition of the lipogenic enzymes by exogenous fatty acids may be the cause, but it should be noted that the livers in the animals fed the FF diet synthesize amounts of these compounds in excess of that reported to inhibit lipogenesis by dietary fatty acids (8,9,26), yet did not exhibit inhibition of fat synthesis. If the liver can distinguish synthesized from absorbed fatty acids, the mechanism is unknown. However, the absorption process in the intestines might provide appropriate signals to the liver, for example, the apoproteins, such as apoB-48 (27,28), synthesized in the intestines and incorporated into the chylomicrons and very low density lipoprotein (VLDL) during fat absorption (29,30).

Liver may accumulate triglycerides because of inadequate synthesis of phospholipids in the livers of animals fed a polyunsaturated fatty acid-deficient diet. Phospholipids are required for the formation of the phospholipoprotein coat (30,31) of the nascent VLDL particles that are secreted by the liver and transport de novo synthesized triglycerides to peripheral tissues (33,34). Dietary corn oil would provide polyunsaturated fatty acids for phospholipid synthesis but the hydrogenated soybean oil diet would not. Yet the liver seems to clear the accumulated triglycerides easily once saturated fatty acids are added to the diets. Thus, one may have to invoke the concept of signals from the intestine during fat absorption, as discussed above, to explain this phenomenon.

The phospholipid and cholesterol content of the liver in all three groups of animals showed little or no change during the course of the experiment. Reiser et al. (12) reported an increase in liver cholesterol in animals fed a diet containing safflower oil for two weeks, but they did not give a time course for this increase; presumably it occurred after 48 hr. Earlier work by Hill et al. (11) also indicated an increase in the synthesis of liver cholesterol in fat-fed rats but with a definite lag period, which is consistent with the observations reported here.

A rapid change in the fatty acid composition of liver in rats fed a FF diet after being starved for 48 hr was demonstrated previously (14). This work demonstrates that the pattern established at 48 hr does not change significantly when the animals are maintained on the FF diet for another 48 hr, except that the palmitic acid level increased from 26 to 31% of the total fatty acids.

The failure of the liver to accumulate stearic acid may have been due to its rapid desaturation to oleic acid (35-37), its conversion to glucose for energy metabolism (38,39), its malabsorption in the gut (see discussion of this point below) or a combination of these factors.

It is generally accepted that the fatty acid composition of the plasma reflects the composition of the fat consumed (30,40). In animals on a fat-free diet, the plasma fatty acid composition is similar to that found in the liver (14). The

plasma fatty acid composition of rats fed the saturated fat diet did not change substantially from that observed in animals fed a FF diet. It may be that because stearic acid is very poorly absorbed (41,42), the lack of marked changes in the stearic acid level reported here for both liver and plasma are indicative of malabsorption (43) rather than differences in the metabolism of the saturated fat. Examination of the feces of the animals on the saturated fat diet indicated appreciable fat was excreted unabsorbed, although the actual amount of fat absorbed by each animal was not measured. Food intake was recorded, and all animals consumed about the same number of calories.

The data presented here suggest that intestinal absorption and transport of exogenous fatty acids have an important role in the regulation of fatty acid metabolism in the liver. Further research, however, will be necessary to prove the existence of any intestinal involvement in the regulation of liver fatty acid metabolism.

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The Effect of Mengovirus Infection on Lipid Synthesis in Cultured Ehrlich Ascites Tumor Cells

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The concept of generally increased lipid synthesis during the initial 2/3 of picornaviral infectious cycles, held by several authors, needs differentiation. In mengovirus-infected Ehrlich ascites tumor cells, an increase in the rate of synthesis of phosphatidylcholine could be confirmed, but for phosphatidylethanolamine constant to decreasing rates of synthesis were found. Moreover, phosphatidylinositol was increasingly synthesized in the midst of the infectious cycle. The changes observed might have their functional expression in the proliferation of smooth cytoplasmic membrane systems that provide the structural framework for the replication of picornaviral RNA and virus assembly. The alterations in the labeling patterns of phosphatidylinositol, phosphatidylglycerol and diphosphatidylglycerol late in virus infection point to increased turnover of these compounds, possibly mediated by phospholipase D. The formation of lysophosphatidylcholine (cytolytic effect) and bis(monoacylglyceryl)phosphate in the final phase of the infectious cycle might be correlated with the liberation of lysosomal enzymes and the development of the cytopathic effect.
Lipids 22, 95-103 (1987).

Infection of susceptible animal cells with picornaviruses causes rapid inhibition of host protein and nucleic acid synthesis while viral RNA is replicated and translated (1). These macromolecular processes have been the subject of extensive studies, whereas the role of cellular membranes in the replicative cycle of picornaviruses has attracted comparably little attention.

Well-documented effects of picornaviral infections at the membrane level are stimulated incorporation of choline into phospholipids and extensive proliferation of smooth endoplasmic membranes (2-10), at a time postinfection when cellular macromolecular synthesis has largely been shut off (1,11,12).

Replication and translation of picornaviral RNA as well as maturation of virus particles are associated with cytoplasmic membranes (13-21). Studies performed by Caliguiri and Tamm (22-24) have identified smooth cytoplasmic membranes as the site of replication and rough cytoplasmic membranes as the site of translation of viral RNAs. Generally, the different phases of the infectious cycle of picornaviruses appear to be dependent on preexisting and newly synthesized cellular membranes.

This communication presents a more detailed investigation of the lipid metabolism of Ehrlich ascites tumor (EAT) cells infected with mengovirus.

MATERIALS AND METHODS

[^{32}P]Orthophosphoric acid was purchased from New England Nuclear (Dreieich, Federal Republic of Germany); [2-^{3}H]myoinositol (9.3 Ci/mmol), [2-^{3}H]glycerol (500 mCi/mmol) and [9,10(n)-^{3}H]oleic acid (2.1 Ci/mmol) were from Amersham Buchler (Amersham, United Kingdom). The solvents in which the radiochemicals were delivered were evaporated under nitrogen. [^{3}H]Glycerol was dissolved in minimum essential medium (MEMS) and [^{3}H]oleic acid was taken up in serum-free MEMS. Lipid standards were obtained from Fluka AG (Buchs, Switzerland), Serva (Heidelberg, Federal Republic of Germany), Sigma (St. Louis, Missouri) and Applied Science Europe B.V. (Oud-Beijerland, The Netherlands). Thin layer chromatography (TLC) and high performance TLC precoated plates (silica gel) as well as all solvents (analytical grade) and spray reagents were from Merck (Darmstadt, Federal Republic of Germany).

Growth of EAT cells and mengovirus. EAT cells were grown in suspension culture at 37 C, essentially according to the procedure described by Van Venrooij et al. (25) as modified by Egberts et al. (26). Mengovirus was grown in monolayers of L 929-cells as performed by Egberts et al. (26).

Mengovirus infection of EAT cells. At a cell density of 5 to 5.5 × 10^{5} cells/ml (logarithmic phase), EAT cell cultures were divided into halves, and the cells were harvested by centrifugation at 225 × g for 5 min at 37 C. The supernatants were saved and the pelleted cells resuspended in a fraction of the corresponding supernatants to result in 20-fold concentrated cell suspensions. Prewarmed mengovirus solution was added to one-half of the cell culture to yield a multiplicity of infection of 50 PFU/cell (unless otherwise stated). The other half was correspondingly mixed with virus-free medium (mock infection).

The mock- and mengovirus-infected cell suspensions were stirred at 37 C for 45 min. Both concentrates were then diluted, with the supernatants kept at 37 C during the time of virus adsorption. Incubation at 37 C was continued for various times when aliquots of the cell suspensions were withdrawn for the incorporation of radioactively labeled lipid precursors and the preparation of lipid extracts as indicated in the figures of the Results section. Lysis of infected cells was followed by the dye exclusion test with trypan blue (27) at 8, 12 (24) hr postinfection.

Incorporation of radioactively labeled precursors into the lipids of mock- and mengovirus-infected EAT cells. EAT cells from 1.2-l cultures were infected as described above. At various times postinfection, 25-ml portions were taken from the mock- and virus-infected cell cultures and centrifuged at 225 × g for 5 min at 37 C; 1.25-ml aliquots were withdrawn from 20-fold concentrated cultures at 0 and 30 min after infection. The cells were washed with 10 ml MEMS and resuspended in 1.25 ml MEMS lacking phosphate, inositol or fetal calf serum in case of labeling with radioactive orthophosphoric acid, myoinositol or oleic acid. For radioactive labeling, 250 μCi [^{32}P]orthophosphoric acid, 250 μCi [2-^{3}H]glycerol, 50 μCi [2-^{3}H]myoinositol and 50 μCi [9,10(n)-^{3}H]oleic acid, respectively, were added in 50-μl volumes to the cell suspensions, which

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were incubated with shaking at 37 C for 15 min (labeling with [2-^{3}H]glycerol: 5 min). Thereafter, the cells were collected by centrifugation at 360 × g for 5 min at 0 C and, in the case of labeling with oleic acid, were washed three times with 20 ml cold, serum-free MEMS that was supplemented with 2.5 mg/ml bovine serum albumin. In the other cases, the cells were washed once with 20 ml cold phosphate-free, inositol-free and complete MEMS, respectively. Finally, the cells were washed twice with 20-ml and 7-ml portions of cold Tris-saline (0.01 M Tris-acetate, pH 7.5 [20 C], 0.15 M NaCl). After centrifugation at 800 × g and 0 C for 5 min, the pellets were stored at −80 C.

Extraction of lipids. Unless otherwise stated, all operations were carried out at 0-2 C. The pelleted cells were extracted three times with 20-vol portions of chloroform/methanol (2:1, v/v) according to Folch et al. (28). The organic phases were evaporated under reduced pressure at 37 C, and the lipids were taken up in chloroform/methanol (2:1, v/v). When EAT cells had been labeled with myoinositol, they were additionally extracted four times with ethanol/distilled water (50:50, v/v). After centrifugation at 2600 × g for 5 min at 4 C, the combined supernatants were evaporated under reduced pressure at 50 C, and the residues were redissolved in ethanol/distilled water (50:50, v/v).

Determination of lipid phosphorus and cholesterol. The method of Chen et al. (29) was modified to achieve quantitative lipid hydrolysis and a higher sensitivity of lipid detection, as described by Schimmel (30). The cholesterol content of lipid extracts was measured by means of a test combination (Boehringer Mannheim, Mannheim, Federal Republic of Germany) according to Röschlau et al. (31).

TLC separation of lipids. Neutral lipids were separated as described by Schimmel (32). By raising the polarity of the solvent system used by Skipski et al. (33) to chloroform/methanol/glacial acetic acid/distilled water (52.5:50:4:1, v/v/v/v), good separation of the phospholipid classes, including a reliable resolution of phosphatidylserine and phosphatidylinositol, was achieved. For the separation of diphosphatidylglycerol and phosphatidylglycerol, the basic solvent system chloroform/methanol/n-propylamine/distilled water (26:12:1.1:1.65, v/v/v/v) was used. The separation of phospholipids was usually performed on HPTLC plates (20 × 10 cm). Lipid classes were identified by cochromatography of extracts with standards in different solvent systems and by detection with specific spray reagents (34,35). Identification of individual lipids was carried out by two-dimensional TLC using combinations of different acidic and basic solvent systems. Lipids were routinely made visible by exposure of the TLC plates to iodine vapor.

Identification of bis(monoacylglyceryl)phosphate. A lipid extract from EAT cells (250 ml cell culture) pulse-labeled with [9,10(n)-^{3}H]oleic acid for 15 min at 7.5 hr postinfection was separated on two HPTLC plates (20 × 10 cm) in the modified solvent system described above. Bis(monoacylglyceryl)phosphate (R_f = 0.76) was eluted with chloroform/methanol (2:1 and 1:2, v/v). Half of the eluate was evaporated and the residue hydrolyzed according to Dawson (36, micromodification of method I). The water-soluble products were separated as described by Poorthuis and Hostetler (37). Glycerol and glycerol-3-phosphate as well as phosphatidylglycerol, diphosphatidylglycerol and the corresponding deacylated products (36) were used as reference substances. The residue of the other half of the eluate was taken up in 10 μl chloroform/methanol (2:1, v/v) and subjected to two-dimensional TLC according to Poorthuis et al. (38) with diphosphatidylglycerol as a reference.

Identification of polyphosphoinositides. EAT cells (450 ml cell culture) were pulse-labeled with 900 μCi [2-^{3}H]myoinositol for 15 min at 2 hr postinfection. After lipid extraction according to Folch et al. (28), the cell residues were additionally extracted with aqueous ethanol as described above. Following evaporation, the residue of the extract was taken up in 500 μl aqueous ethanol, and 400 μl of the resulting solution was separated semipreparatively on TLC plates using the solvent system described by Eberlein and Gercken (39). After localization of the compounds by means of a Beta-camera (Laboratorium Prof. Dr. Berthold, Wildbad, Federal Republic of Germany), they were successively eluted with chloroform/methanol (2:1, 1:1 and 1:2, v/v, respectively). The eluates were evaporated and the residues hydrolyzed (36). The deacylation products were separated by TLC according to Koch-Kallnbach and Diringer (40). Glycerophosphoinositol derived from phosphatidylinositol (36) was used as a reference compound. The remainder of the aqueous ethanol extract was separated in the solvent system described by Eberlein and Gercken (39) using phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-diphosphate for reference.

Sucrose density gradient centrifugation of cell homogenates. Two 1.2-l EAT cell cultures were mock- and mengovirus-infected (multiplicity of infection: 100 PFU/cell), and the cells were harvested at 6 hr postinfection. They were pulse-labeled with 246 μCi of [2-^{3}H]myoinositol for 5 and 15 min. Cell rupture and sucrose density gradient centrifugation of the homogenates were carried out essentially as described by Nelson et al. (41).

Measurement of radioactivity. Radioactivity was measured by liquid scintillation counting after adequate pretreatment of samples. Radioactively labeled lipids were localized on TLC plates by means of a Beta-camera (LH 290 HR, Berthold), by a TLC Linear Analyzer (LB 282, Berthold) and by autoradiography.

RESULTS

Figure 1 shows the incorporation of [2-^{3}H]glycerol into total lipids of mengovirus- and mock-infected EAT cells. In virus-infected cells, enhanced incorporation of the radiolabel was observed between 1.5 and 4.5 hr postinfection, followed by a sharp decrease, whereas incorporation in mock-infected cells increased in parallel with cell density.

It should be noted that for the comparison of lipid syntheses in virus- and mock-infected cells, in the latter case linearly growing cultures were used as controls. Since in the following the incorporations of radioactively labeled precursors into individual lipid species are expressed as percentage distributions, deviations from constancy during mock-infection should allow the detection of possible disturbances or inhomogeneities in the formation of these lipids. As demonstrated in Figures 2 to 5 and Figure 7, such deviations were not observed to any significant extent during the entire period of measurement. The averages of the relative incorporations of precursors into

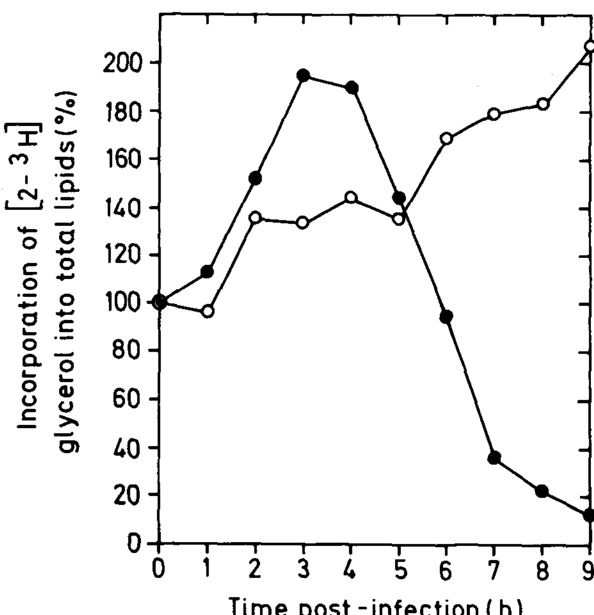

FIG. 1. Incorporation of [2-^{3}H]glycerol into total lipids of mock-infected (○) and mengovirus-infected (●) EAT cells (5 min pulse/25 min chase). Incorporation prior to infection is set equal to 100%. The values represent the means of two individual experiments. The analytical determinations were run as duplicates.

individual lipids at different time points of mock-infection should therefore serve as the most reliable controls.

The percentage distribution of the incorporation of [2-^{3}H]glycerol into various phospholipid classes of mock- and mengovirus-infected EAT cells is depicted in Figure 2. The rate of synthesis of phosphatidylcholine increased up to about four times the initial value until 3 hr postinfection and fell back to about this value until 9 hr postinfection. The rate of synthesis of phosphatidylethanolamine, however, decreased gradually during the infectious cycle as compared to the control. For phosphatidylinositol, a significantly enhanced rate of synthesis was found in the middle of the infectious cycle. The same results were obtained after labeling the phospholipids with [^{32}P]orthophosphoric acid (30).

However, phosphatidylglycerol and diphosphatidylglycerol showed different labeling patterns after incorporation of [2-^{3}H]glycerol and [^{32}P]orthophosphoric acid, respectively (30). Whereas labeling was increased late in the infectious cycle in the former case, it was reduced in the latter case. For both phospholipids, the possibility of multiple labeling has to be considered, especially when the labeling is carried out with glycerol. Labeling with [2-^{3}H]glycerol offered information about the synthesis of those phospholipid classes that did not show sufficient incorporation during labeling with [^{32}P]orthophosphoric acid. Increased synthesis was also observed for phosphatidylserine (with maxima at 1 and 8 hr postinfection) (Fig. 2d), whereas a continuously low incorporation was found for lysophosphatidylcholine (Fig. 2a) and for sphingomyelin (Fig. 2b). Although the latter compound is not a glycerophospholipid, its labeling can be explained by metabolization of glycerol. For bis(monoacylglyceryl) phosphate, an enhanced incorporation was noted in the second half of the infectious cycle (Fig. 2h).

A study of the incorporation of [9,10(n)-^{3}H]oleic acid into the lipids of mock- and mengovirus-infected EAT cells has shown that about 80% of the maximal labeling occurred during the initial 15 min after the addition of the radiochemical (30). Thus, a sufficiently high labeling was to be expected from the performance of pulse labeling for 15 min; this also made possible the analysis of the radioactively labeled neutral lipid- and glycolipid-fractions.

The percentage distribution of the incorporation of [9,10(n)-^{3}H]oleic acid into the phospholipid fractions of mock- and mengovirus-infected EAT cells, delineated in Fig. 3, essentially confirmed the results reported above. Again, constant incorporation rates were observed for the control cultures. The cultures infected with mengovirus revealed differentiated incorporation patterns for the different phospholipid classes. Oleic acid incorporation into phosphatidylcholine (Fig. 3c) was enhanced until 3 hr postinfection to an extent comparable to that observed with glycerol (Fig. 2c). Subsequently, the rate of synthesis dropped rather steadily until the end of the infectious cycle (insert, Fig. 2c). An incorporation only slightly diminished was observed for phosphatidylethanolamine (Fig. 3f), whereas the incorporation of [2-^{3}H]glycerol (Fig. 2f) and [^{32}P]orthophosphoric acid (30) into this phospholipid was substantially reduced. The increased production of phosphatidylinositol (Fig. 3e) observed between 4 and 6 hr postinfection correlated with the results obtained with [2-^{3}H]glycerol (Fig. 2e). Increased synthesis of this phospholipid between 4 and 5 hr postinfection could also be demonstrated by incorporation of phosphate label (30).

Bis(monoacylglyceryl)phosphate was determined together with diphosphatidylglycerol and phosphatidylglycerol, as in this series of experiments its localization on the chromatograms could not be achieved with confidence. Between 6 and 9 hr postinfection, an increase in the incorporation of radiolabel into the three phospholipids was observed (Fig. 3g) comparable to that found after labeling with [2-^{3}H]glycerol (Figs. 2g, 2h) with respect to its extent as well as its temporal appearance. The synthesis of lysophosphatidylcholine, sphingomyelin and phosphatidylserine (Figs. 3a, 3b, 3d) was slightly enhanced between 6 (or 7) and 9 hr postinfection.

The synthesis of most neutral lipids (2-monoacylglycerol, 1-monoacylglycerol, cholesterol, 1,3-diacylglycerol) was more or less increased after mengovirus infection or significantly decreased in the case of cholesterol esters (30). Because of the importance of 1,2-diacylglycerols and triacylglycerols as precursors of phospholipids, only their labeling characteristics are presented in Figure 4. The incorporation of [^{3}H]oleic acid into 1,2-diacylglycerols was decreased throughout the entire infectious cycle, whereas the formation of triacylglycerols was significantly enhanced from 0 to 4.5 hr postinfection.

The increased labeling of phosphatidylinositol with [^{32}P]orthophosphoric acid (30) and [2-^{3}H]glycerol (Fig. 2e) in the middle of the infectious cycle necessitated further examination, especially in consideration of the numerous publications dealing with increased turnover of phosphatidylinositol (e.g., 42–47). The incorporation of [2-^{3}H]myo-inositol into the lipids of mock- and mengovirus-infected EAT cells is shown in Figure 5. In mengovirus-infected cells, its incorporation into phosphatidylinositol increased

dramatically between 4.5 and 9 hr postinfection, up to about 14-fold as compared with the mock-infected controls at 7 hr postinfection. The label was not metabolized and thus was incorporated exclusively into phosphatidylinositol, as demonstrated by cochromatography of the lipid extracts with phosphatidylinositol standard in the acidic solvent system (30).

Nearly equal amounts of tritium label (mainly as myoinositol; compare ref. 40) were found within the initial 3 hr postinfection in the aqueous phases after lipid extraction (28) of mengovirus-infected EAT cells. The amount of tritium label diminished distinctly between 3 and 4 hr postinfection and remained at a nearly constant, low level (Fig. 6), indicating that uptake of inositol was drastically reduced after about 4 hr postinfection and/or that the polyalcohol was increasingly consumed in the process of phospholipid synthesis.

Extraction of the cell pellets with aqueous ethanol after removal of chloroform/methanol-soluble lipids yielded another fraction of phosphoinositides. By TLC separation of the ethanolic extracts, the following phosphoinositides could be characterized by their R_f values: phosphatidylinositol (PI), 0.48; unknown phosphoinositide (PI-X), 0.41; phosphatidylinositol-4-phosphate (PI-4-P), 0.28; lysophos-

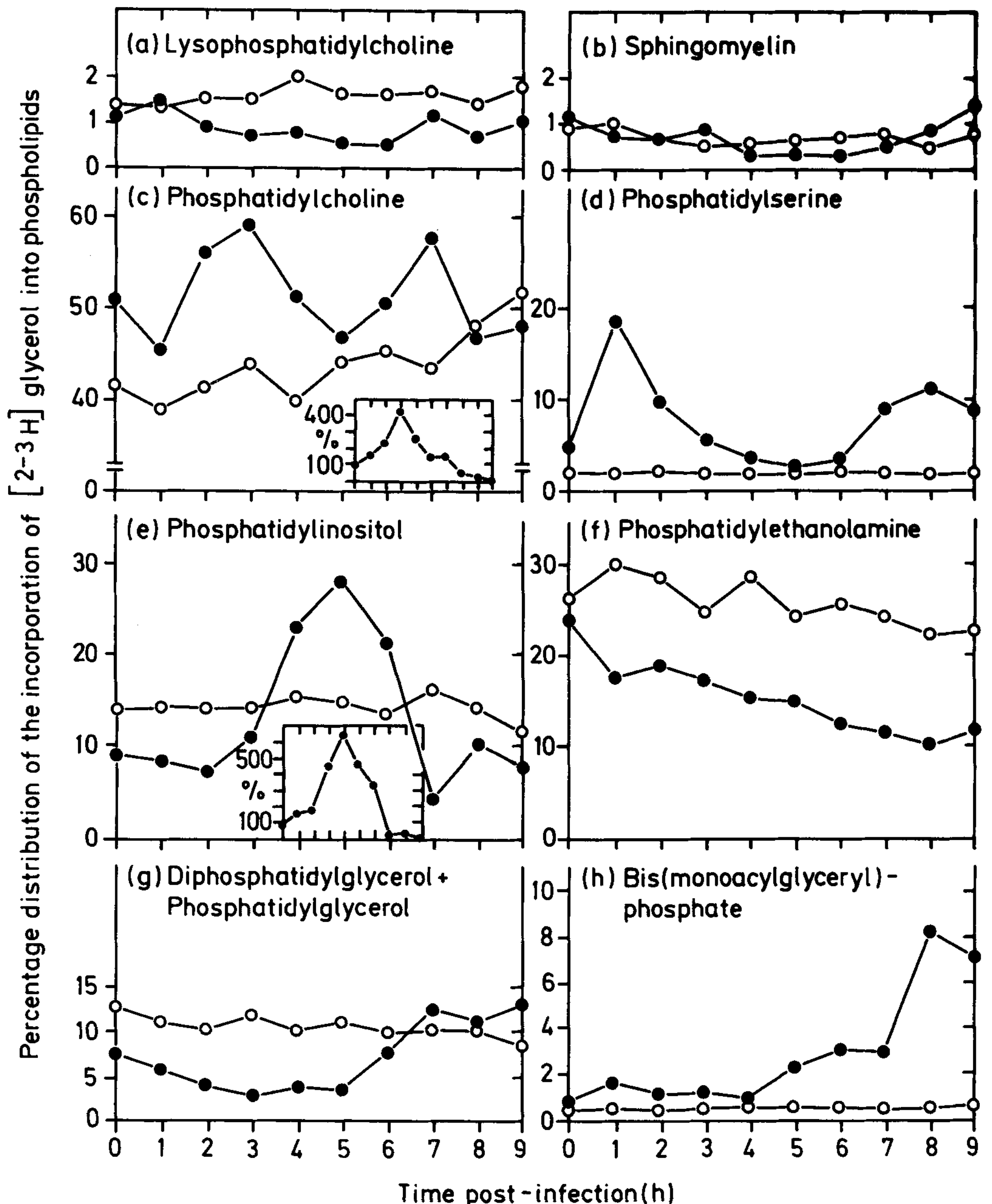

FIG. 2. Incorporation of [2-^{3}H]glycerol into the phospholipids of mock-infected (○) and mengovirus-infected (●) EAT cells (5 min pulse/25 min chase). The values represent the means of two individual experiments. The analytical determinations were run as duplicates. The inserts in panels c and e show the alterations of the respective incorporation rates (incorporation rates at 0 hr postinfection are set equal to 100%).

phatidylinositol; 0.21; phosphatidylinositol-4,5-diphosphate (PI-4,5-DP), 0.08. The R_f values of the deacylation products of PI, PI-X, PI-4-P and PI-4,5-DP were 0.30, 0.27, 0.20 and 0.09, respectively. In consideration of its chromatographic behavior, the unidentified component (PI-X) is assumed to contain a sugar moiety different from or in addition to inositol. Figure 7 illustrates that the incorporation of [2-^{3}H]myoinositol into ethanol/water-soluble phosphoinositides of mengovirus-infected EAT cells was increased about 22-fold at 2 hr postinfection as compared to mock-infected cells.

To gain information on the intracellular localization of

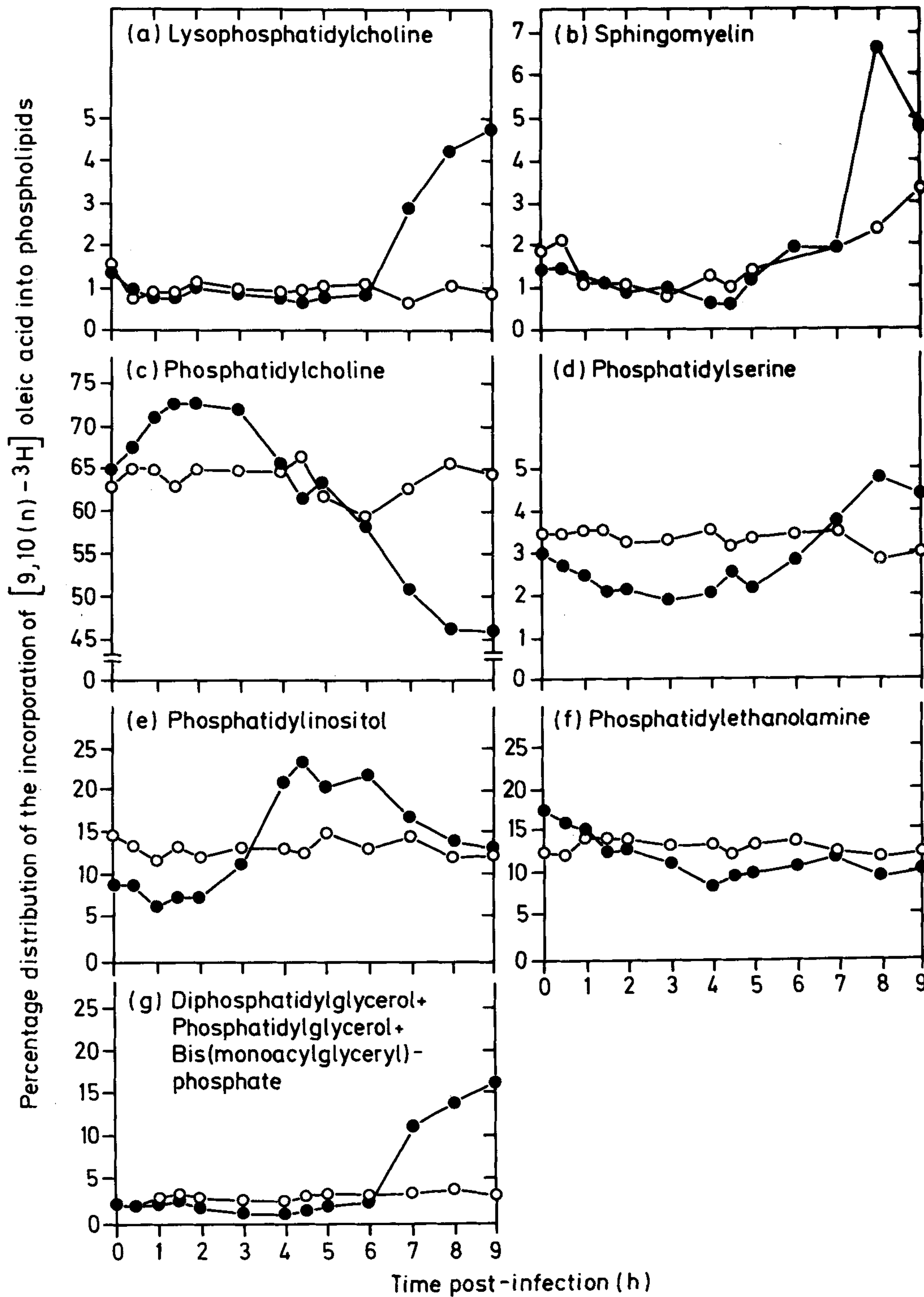

FIG. 3. Incorporation of [9,10(n)-^{3}H]oleic acid into the phospholipids of mock-infected (○) and mengovirus-infected (●) EAT cells (15 min pulse). The values represent the means of two individual experiments. The analytical determinations were run as duplicates.

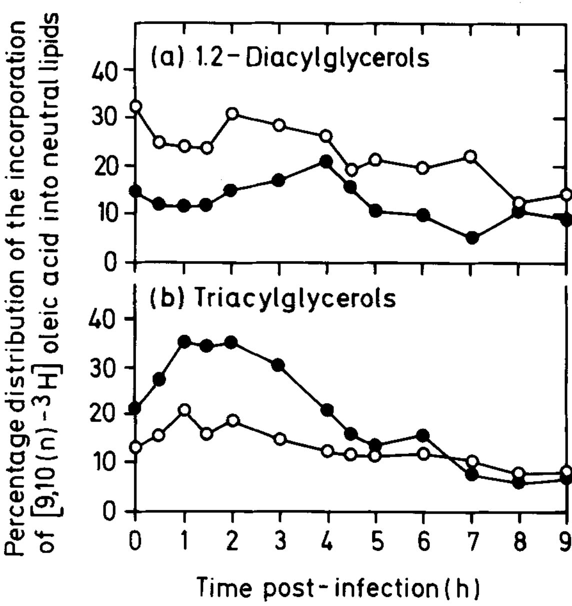

FIG. 4. Incorporation of [9,10(n)-^{3}H]oleic acid into the phospholipids of mock-infected (○) and mengovirus-infected (●) EAT cells (15 min pulse). Only the data obtained for 1,2-diacylglycerols and triacylglycerols are presented. The values represent the means of two individual experiments. The analytical determinations were run as duplicates.

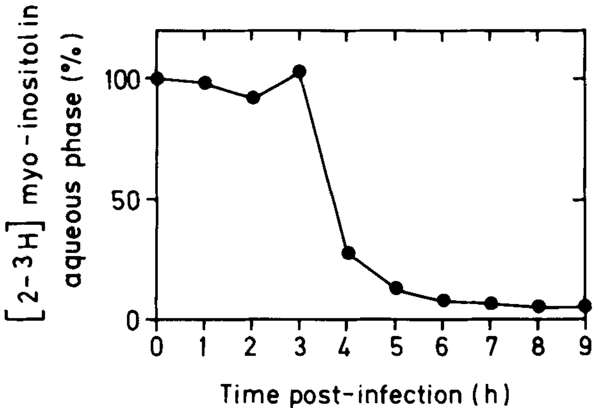

FIG. 6. Alterations of ^{3}H-label (mainly free inositol) in the aqueous phases of extracts (according to Folch et al., 28) of mengovirus-infected EAT cells after 15 min pulse-labeling with [2-^{3}H]myoinositol. The radioactivity in the aqueous phase (60 195 cpm from 0.5 ml cell suspension) measured at the beginning of the infection is set equal to 100%. The values represent the means of two individual experiments. The analytical determinations were carried out fivefold.

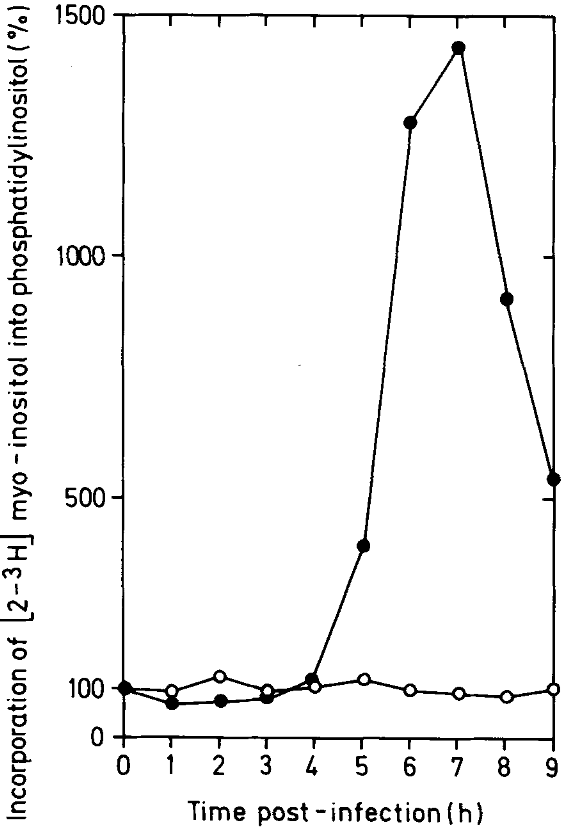

FIG. 5. Incorporation of [2-^{3}H]myoinositol into phosphatidylinositol of mock-infected (○) and mengovirus-infected (●) EAT cells (15 min pulse). Incorporation prior to infection is set equal to 100%. The values represent the means of two individual experiments. The analytical determinations were run as duplicates.

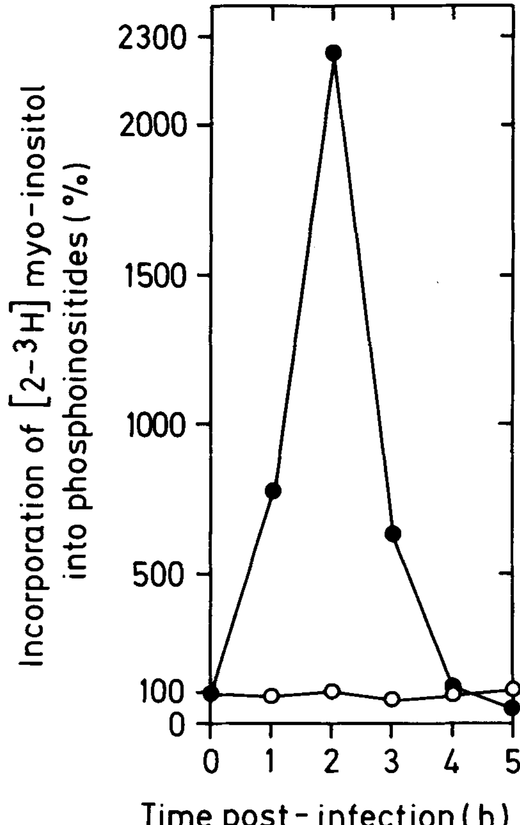

FIG. 7. Incorporation of [2-^{3}H]myoinositol into phosphoinositides of mock-infected (○) and mengovirus-infected (●) EAT cells (15 min pulse). After extraction according to Folch et al. (28) of the cells, the residues were extracted with aqueous ethanol.

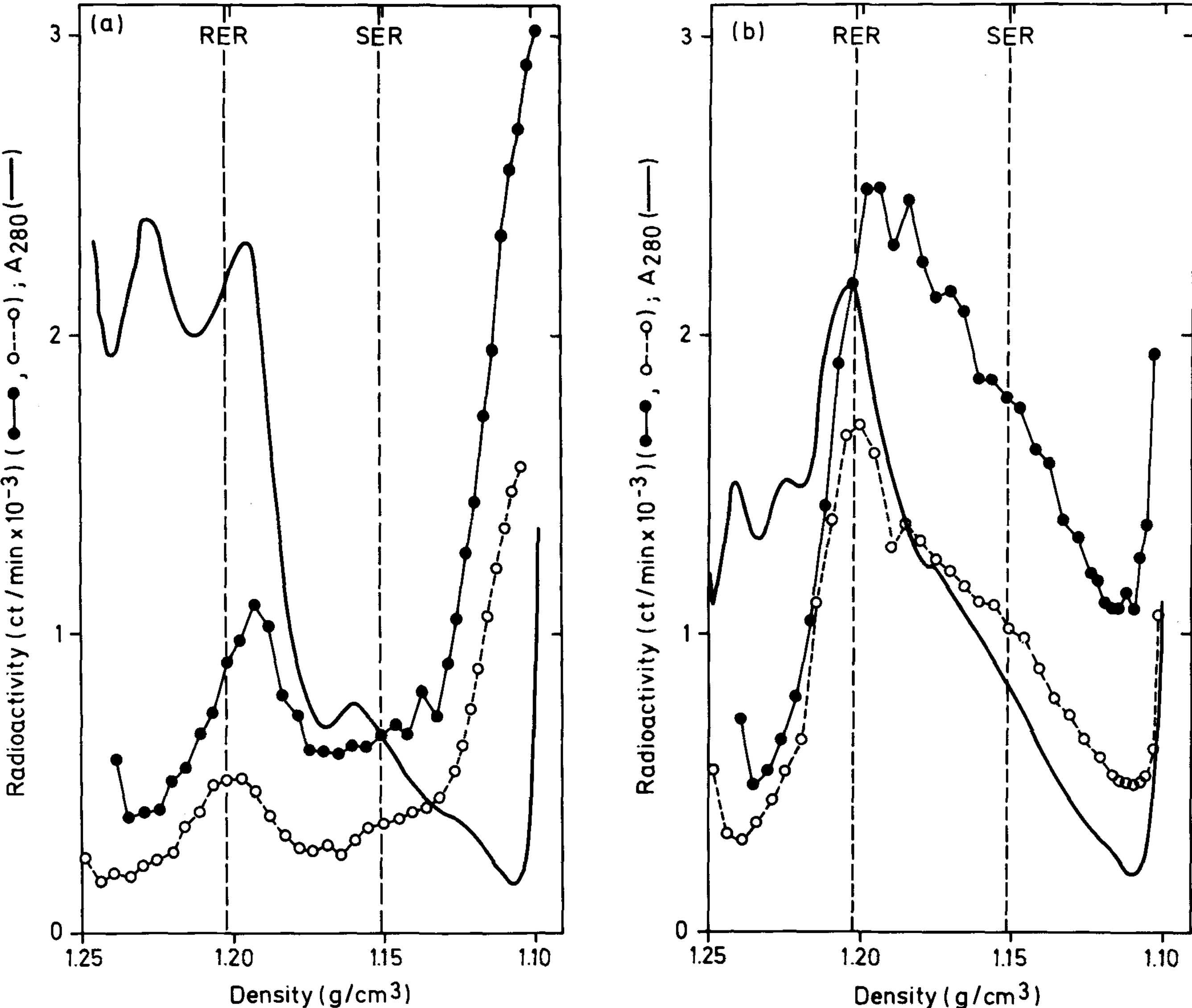

FIG. 8. Intracellular localization of the incorporation of [2-^{3}H]myoinositol into phosphatidylinositol of mock-infected (a) and mengovirus-infected (b) EAT cells. Mock- and mengovirus-infected EAT cells were homogenized in a hypotonic Tris buffer, pH 8, and the postnuclear supernatants were subjected to sucrose density gradient equilibrium centrifugation according to Nelson et al. (41). ○, 5 min pulse and ●, 15 min pulse of [2-^{3}H]myoinositol incorporation; ——, optical density at 280 nm. The specific densities for smooth endoplasmic reticulum (SER, 1.1504 g/cm^3) and rough endoplasmic reticulum (RER, 1.2020 g/cm^3) of EAT cells were taken from Nelson et al. (41).

the turnover of phosphatidylinositol, homogenates of mock- and mengovirus-infected EAT cells pulse-labeled with [2-^{3}H]myoinositol for 5 and 15 min, respectively, were subjected to sucrose density gradient equilibrium centrifugation. A comparison of Figures 8a and 8b shows increased incorporation of the label in virus-infected cells. The shape of the radioactivity profiles after pulse-labeling for 5 min did not differ significantly from that obtained after pulse-labeling for 15 min. The radioactivity profiles of the homogenates derived from mengovirus-infected EAT cells revealed a minor shoulder at the density of the smooth endoplasmic reticulum, however.

DISCUSSION

On the basis of the present results, the previously established concept of generally increased lipid synthesis during the course of picornaviral infections of mammalian cells (2,5–7,48,49) could be further differentiated. A study of the effect of mengovirus infection on lipid synthesis in EAT cells (50) has shown that in infected cells the lipid phosphate content increased steadily up to 6 hr postinfection to nearly 130% of the initial value, then dropped to about 90% within 1 hr and remained at this level. Similar results were obtained for the cholesterol content. Using [^{32}P]orthophosphoric acid, [2-^{3}H]glycerol and [^{3}H]oleic acid as radioactively labeled lipid precursors, this net increase in lipid synthesis was demonstrated to be differentially distributed among the most important phospholipid classes. During the first half of the infectious cycle, increased synthesis of phosphatidylcholine was observed, but for phosphatidylethanolamine constant to decreasing rates of synthesis were found. Phosphatidylinositol showed an enhanced rate of synthesis that was limited

to the middle of the infectious cycle between 4 and 5 hr postinfection. Increased incorporation of labeled myo-inositol into phosphatidylinositol was also shown to occur in poliovirus-infected human embryonic lung cells (50).

It is remarkable that increased new synthesis of phosphatidylinositol temporally coincides with the proliferation of smooth cytoplasmic membranes as observed by electron microscopy (23,24). It is known that replication of picornaviral RNA is associated with smooth internal membrane systems (17,22–24), while the synthesis of viral proteins is dependent on rough cytoplasmic membranes (23,24). In this connection, it is pertinent to note that mengovirus-infected EAT cells exhibit maximal viral RNA synthesis between 5 and 6 hr postinfection (Schimmel, H., and Traub, P., unpublished results). For comparison, viral protein synthesis reaches its maximum between 3 and 4 hr postinfection in this system (26). Interestingly, sucrose density gradient centrifugation of homogenates obtained from mengovirus-infected EAT cells revealed that newly synthesized phosphatidylinositol is eventually incorporated into membranes with the density of rough and smooth endoplasmic reticulum. The mengovirus-induced changes in lipid synthesis of EAT cells might also be correlated with the dramatic alterations in membrane permeability that set in 3 hr postinfection (51) and with the shut-off of host-specific protein synthesis in the first half of the infectious cycle (26).

Increased synthesis of bis(monoacylglyceryl)phosphate, a marker lipid of secondary lysosomes (52), was observed during the last third of the infectious cycle. This might be correlated with the liberation of lysosomal enzymes, as was found during the development of the cytopathic effect in different cell lines infected with picornaviruses (37,53,54). Furthermore, phosphatidylinositol, increasingly synthesized in the middle of the infectious cycle, is known to stimulate the formation of bis(monoacylglyceryl)phosphate from phosphatidylglycerol (55). The enhanced incorporation of glycerol and oleic acid labels, but not of phosphate label, into phosphatidylglycerol and diphosphatidylglycerol late in infection indicates an increased participation of these phospholipid species in turnover reactions; in this case, phospholipase D-catalyzed transfer reactions (56,57) might play an important role. It is reasonable to assume that the changes in lipid synthesis late in infection contribute to the destruction of the plasma membrane and thus to the release of progeny virus particles into the medium. Mengovirus-infected EAT cells start to become permeable to trypan blue 6 to 7 hr post infection (51). While intracellularly the first infectious mengovirus particles can be detected 5 hr postinfection, extracellularly the first virus particles appear 8 hr post-infection (51).

Another striking observation of the present investigation was the enhanced turnover of phosphatidylinositol late in infection. It could be shown to be localized to rough cytoplasmic membranes and to temporally coincide with the most active phase of virus assembly (51). Its mechanism appears different from that of phosphatidylinositol turnover in connection with virus-receptor interactions (58) and receptor-mediated endocytosis (46,47). In this case, as formulated by Michell (42,43), Nishizuka (46) and Berridge and Irvine (47), enhanced labeling with phosphate catalyzed by diglyceride kinase should have been expected, whereas in the late phase of mengovirus infection of EAT cells only the inositol moiety of phosphatidylinositol was exchanged. For the decomposition reaction, a phospholipase D activity has to be postulated. Enzyme activities of this type have been demonstrated in bacteria and plants (57), but only rarely in animal cells (45,46). However, a respective reaction sequence for phosphatidyl turnover has been discussed by Hokin-Neaverson (59).

Finally, the finding that 2 hr postinfection substantially increased incorporation of myoinositol into ethanol/water-soluble phosphatidylinositol occurs indicates the existence of a second cellular pool of the phospholipid in mengovirus-infected EAT cells. Two different cellular pools of phosphatidylinositol have already been demonstrated in synapses (60), in microsomal membranes of rat liver (61) and in a tumor cell line (62).

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Measurement of Free and Bound Malondialdehyde in Vitamin E-Deficient and -Supplemented Rat Liver Tissues[1]

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The quantity of free malondialdehyde (MDA) in liver tissues of rats fed vitamin E-deficient or -supplemented diets for 43 wk was measured by a newly developed high performance liquid chromatographic (HPLC) method. Bound MDA was quantified by the same HPLC method after alkaline hydrolysis of tissue homogenates. Tissues from vitamin E-deficient animals showed levels of free MDA about 15 times higher but levels of bound MDA less than 2 times higher than the vitamin E-supplemented animals. Free MDA is the major form in vitamin E-deficient tissues, but bound MDA is predominant in vitamin E-supplemented tissues. Conventional thiobarbituric acid (TBA) test results revealed that the content of TBA-reactive substances expressed in MDA equivalents was much higher than the actual free MDA levels in all groups. Results indicate that free MDA level measured by HPLC is a more sensitive index than the TBA value for lipid peroxidation. Some other TBA-reactive substances seem to exist in liver tissue regardless of the dietary treatment.
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Malondialdehyde (MDA), one of the most-studied products of lipid peroxidation, generally has been regarded as involved in causing cell damage and in the formation of lipofuscin age pigments (1-3). It is also well documented that MDA undergoes reactions with amino acids (4-7), proteins (8-14) and nucleic acids (15,16) and can be metabolized (17). The condensation product of MDA with the primary amino groups of biomolecules has been known to possess fluorescence due to a Schiff base-type structure. The crosslinking of biomolecules with MDA under physiological conditions is of great biochemical and physiological interest.

A common method to measure the degree of oxidative damage in biological and food systems is the thiobarbituric acid (TBA) test. It has been suggested by several investigators, however, that this colorimetric method is not specific for MDA, since other known and unknown compounds produce positive reactions (1,18-20). It is questionable, therefore, whether the TBA value can be used as a reliable index for lipid peroxidation. A high performance liquid chromatographic (HPLC) method (21) was recently developed in our laboratory that is specific and sensitive for the direct measurement of MDA in tissues.

There are many reports in the literature on the measurement of MDA using the TBA test for various peroxidizing conditions (20,22-24), but little is known about the actual quantity of MDA produced. In the present study, free MDA levels were measured by HPLC to assess the level of in vivo peroxidation in rat liver tissues from animals fed diets deficient in or supplemented with vitamin E for 43 weeks. Hydrolysis of tissue homogenates was carried out to estimate the amount of MDA generated from the bound form of MDA-biomolecule complexes. The actual MDA levels were compared to the conventional TBA test results.

MATERIALS AND METHODS

Animals and diets. Three groups of eight Sprague-Dawley female weanling rats were fed one of the following three diets for 43 weeks: 1) a modified basal vitamin E-deficient diet developed by Draper et al. (25), adequate in all respects except for vitamin E (−E group); 2) the basal diet supplemented with 30 mg/kg diet of RRR-α-tocopheryl acetate (1.36 IU/mg, type 3, Sigma Chemical, St. Louis, Missouri) (NE group); or 3) the basal diet supplemented with 300 mg/kg diet of RRR-α-tocopheryl acetate (+E group). Diets and water were provided ad libitum. Sodium salt of sulfamerazine (Sigma) was added to drinking water (1 g/3.8 l) to prevent pulmonary infection. After 43 weeks, the animals were killed and the tissues were removed, washed three times with cold physiological saline solution and blotted dry. The tissues were individually wrapped in parafilm and stored at −70 C.

Free MDA determination. A 10% (w/v) liver tissue homogenate in cold 0.01 M sodium phosphate buffer, pH 7.0, was prepared using a Brinkman homogenizer equipped with a PT20ST probe generator (Brinkman Instruments, Westbury, New York). An aliquot of the 4 ml homogenate was immediately filtered through a 50-ml Amicon cell equipped with a YC05 Diaflo ultrafiltration membrane (Amicon Corp., Danvers, Massachusetts) to remove compounds with a molecular weight greater than 300. The Amicon cell was operated under 35 psi pressure of nitrogen gas. The clear filtrate was used for HPLC analysis.

The HPLC separations were performed on a Spherogel-TSK 1000PW size exclusion column, 7.5 mm ID × 30 cm (Beckman Instruments, Berkeley, California) with a mobile phase of 0.1 M sodium phosphate buffer, pH 8.0, at a flow rate of 0.6 ml/min at ambient temperature. The absorbance was monitored at 267 nm.

Bound MDA determination. A 4-ml aliquot of 10% (w/v) liver tissue homogenate was taken into a 10-ml graduated centrifuge tube. The pH of the homogenate was adjusted to 13 using 100 μl of saturated NaOH solution and incubated in a 60 C water bath for 30 min. The hydrolyzed homogenate was neutralized to pH 8.0 with concentrated HCl and immediately subjected to ultrafiltration using the Amicon cell under nitrogen pressure. The clear filtrate was analyzed to quantify MDA by the previously described HPLC method. This value of MDA is referred to as the total MDA of liver tissues. The difference between MDA levels prior to and after hydrolysis is called bound MDA.

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Preparation of MDA standard. MDA was prepared by hydrolysis of 1,1,3,3-tetraethoxypropane (TEP) (Aldrich Chemical Co., Milwaukee, Wisconsin) by a modification of the method of Kwon and Watts (26). One mmol of TEP was dissolved in 100 ml of 0.01 N HCl and heated in a 50 C water bath for 60 min. A standard solution of MDA (1×10^{-5} M) was prepared by diluting 0.1 ml of the hydrolyzed TEP solution to 100 ml with 0.01 M sodium phosphate buffer, pH 7.0. The absorption spectrum of MDA standard solution was obtained with a Beckman Model DU-8 spectrophotometer using a 1-cm quartz cell. The optical density of MDA solution at λmax (267 nm) was measured to verify the molar extinction coefficient and, thus, the purity of the solution.

TBA test. The measurement of TBA reactive substances (TBA-RS) in rat liver tissue was performed by a modified method of Uchiyama and Mihara (20). A 0.5-ml aliquot of 10% (w/v) tissue homogenate in cold 0.01 M sodium phosphate buffer, pH 7.0, was taken into a 20-ml screw-capped test tube. Three ml of 1% phosphoric acid and 1 ml of 0.6% TBA solution were added, and the mixture was heated for 45 min in boiling water bath. After cooling to room temperature, the reaction mixture was extracted with 4 ml of n-butanol. The butanol phase was separated by centrifugation at 2000 rpm for 10 min. The absorbance of the butanol phase was measured at 535 and 520 nm by a Beckman Model DU-8 spectrophotometer. The difference in absorbance was used for the calculation of the amount of TBA-RS. Various concentrations of MDA standard solution were subjected to the same TBA test procedure, and a standard curve for the TBA-MDA complex was prepared. The contents of TBA-RS in the sample tissues were calculated as MDA equivalents from the standard curve.

Tissue α-tocopherol determination. The HPLC method developed by Zaspel and Csallany (27) was used to measure the content of α-tocopherol in rat liver tissues. The HPLC separations were performed on a 10-μm Hibar II Lichrosorb RP-18 column, 250 × 4.6 mm (EM Reagents, Gibbstown, New Jersey), preceded by a guard column, 70 × 4.6 mm, packed with perisorb RP-18 (EM Reagents) with a mobile phase of 2% water in methanol at a flow rate of 2 ml/min. Tissue α-tocopherol was detected by fluorescence at an excitation wavelength of 295 nm and an emission wavelength of 340 nm using a Perkin-Elmer Model 650-10S fluorescence spectrophotometer (Perkin-Elmer Corp., Norwalk, Connecticut) equipped with a 20 μl capacity quartz flow cell. Tissue α-tocopherol concentrations were calculated from peak area responses using a standard curve prepared by chromatography of known amounts of pure d-α-tocopherol.

Statistical analysis of data. Comparisons of three group means were done by ANOVA and Tukey's multiple comparison test (28) and comparisons of two group means by Student's t-test.

RESULTS AND DISCUSSION

Animal body weights and hepatic tissue α-tocopherol concentrations of rats fed vitamin E-deficient or -supplemented diets are shown in Table 1. The vitamin E-deficient group started to show a gradual decrease in growth rate at 22 weeks on the experimental diet compared to the vitamin E-supplemented groups. By 43 weeks of age, when animals were killed, the vitamin E-deficient rats weighed only 76% of the vitamin E-supplemented rats. A significantly high accumulation of α-tocopherol was shown in the liver tissues of vitamin E-supplemented groups. A detectable amount of α-tocopherol was still found in the liver of the −E group after 43 weeks on the completely vitamin E-deprived diet. This small amount of α-tocopherol was derived from inborn tissue storage.

Free and bound MDA concentrations of rat liver tissues following 43 weeks of feeding diets deficient in or supplemented with vitamin E are also shown in Table 1. The amount of free MDA measured in tissues of the −E group was about 15 times higher than NE and +E groups. No difference in the free MDA level was seen between NE and +E groups, even though the +E group had been fed 10 times more vitamin E than the NE group. This seems to indicate that the protective effect of vitamin E against in vivo lipid peroxidation is not dose-dependent, and the normal dietary vitamin E level offers as much protection as the higher level.

Tissue homogenates were hydrolyzed to estimate the amount of MDA present in the bound form with biomolecules such as amino acids, proteins or nucleic acids. After hydrolysis, a considerable amount of MDA was released in all the groups regardless of the dietary treatment. The average amount of released MDA (referred to as bound MDA in Table 1) in the −E group was significantly higher than that in the +E group. No difference was shown between −E and NE groups. It has been found that bound MDA is a predominant form of MDA present in vitamin E-supplemented liver tissue. It is interesting to note that less MDA exists in the bound form than in a free form in the −E liver tissues, indicating

TABLE 1

Body Weights and Concentrations of α-Tocopherol, Free and Bound Malondialdehyde in Rat Liver

Group	Body weight (g) Wk 0	Body weight (g) Wk 43	α-Tocopherol (μg/g tissue)	Free MDA (μg/g tissue)	Bound MDA (μg/g tissue)
−E	56.6 ± 1.28	262.5 ± 14.19^a	3.20 ± 0.65^a	3.91 ± 0.35^a	2.66 ± 0.34^a
NE	52.8 ± 1.61	345.3 ± 7.35^b	36.54 ± 4.08^a	0.28 ± 0.04^b	2.29 ± 0.25^{a,b}
+E	53.7 ± 1.61	341.3 ± 16.19^b	138.38 ± 19.63^b	0.25 ± 0.06^b	1.49 ± 0.27^b

Values represent the mean ± S.E.M. for 8 animals per dietary groups. Means within each column not sharing a common superscript letter are significantly different ($p < 0.05$) by Tukey's multiple comparison test.

possible faster MDA production than the capability to be reactive with some other compounds. The fact that there is no significant difference in bound MDA levels between −E and NE groups in spite of a large difference in free MDA levels might indicate that the highly peroxidative −E tissue could clear toxic free MDA more efficiently than normal tissue by possibly increasing the activity of aldehyde dehydrogenase or by excreting it in the urine (29) as a defense system. Recently, Hjelle and Petersen (30) reported that rat liver contained at least three aldehyde dehydrogenases which oxidize MDA, two with relatively high affinity for MDA in cytosol and one with lower affinity in mitochondria. We have also found a significantly higher level of TBA-RS in vitamin E-deficient rat urine than in urine from supplemented groups (unpublished data).

Comparison of MDA levels to TBA-RS in liver tissues is shown in Figure 1. Free and total (free + bound) MDA concentrations were separately compared to the amount of TBA-reactive substances within each dietary group. Levels of TBA-reactive substances were significantly higher than free MDA in all the dietary groups and also significantly higher than total MDA levels in the −E and +E groups. The vitamin E-deficient group showed 3-4 times more TBA-RS than vitamin E-supplemented groups. The difference in the levels of TBA-RS among the three dietary groups is much less than that for free MDA levels. These results indicate the definite presence of some other TBA-RS that are not MDA in the liver tissues of all dietary groups.

The ratios of TBA-RS as an equivalent of MDA to free and total MDA were calculated (Table 2) to show the relationship between these two lipid peroxidation indices. It was found that the ratio of TBA-RS to free MDA in the −E group was much lower (3.0:1) than the ratios of NE and +E groups (12.9:1 and 12.2:1, respectively). However, the ratios of TBA-RS to total MDA in liver tissues were similar in all dietary groups. These results demonstrate that the proportion of free MDA in TBA-RS measured by the TBA test is much higher in the vitamin E-deficient liver tissues than in the vitamin E-supplemented tissues. However, similar proportions of total MDA exist in TBA-RS in both vitamin E-deficient and vitamin E-supplemented liver tissues, indicating that most of the MDA exists in a bound and not a free form in the vitamin E-supplemented state. The present study also demonstrates that the TBA test may be useful for measuring the peroxidative state of tissues but that it should not be equated with MDA levels.

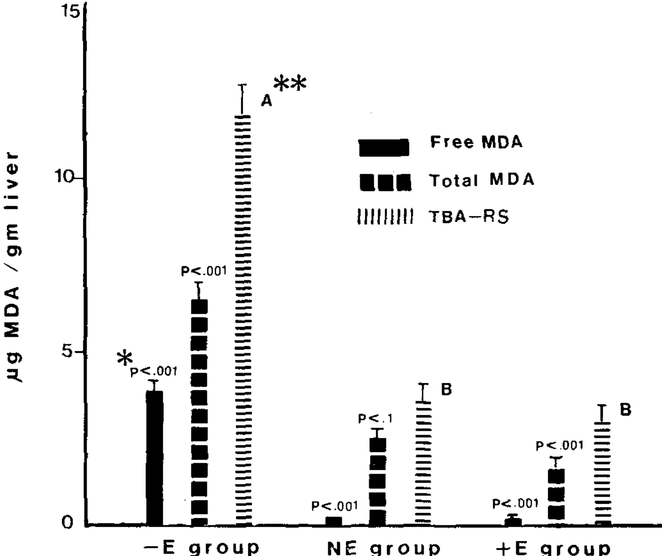

FIG. 1. Comparison of the levels of MDA and TBA-reactive substances. Values represent the mean ± SEM for eight animals for dietary group. *, P values at the top of the MDA bars indicate the results of Student's t-test between free or total MDA levels and the TBA-RS within each dietary group. **, Differences in TBA-RS levels among the dietary groups were tested by Tukey's multiple comparison method. Means not sharing a common letter are significantly different (p < 0.05).

TABLE 2

The Ratios of TBA-Reactive Substances to Free and Total Malondialdehyde[a]

Group	TBA-RS[b]/free MDA	TBA-RS/total MDA
−E	3.0	1.8
NE	12.9	1.4
+E	12.2	1.7

[a]Ratios were calculated from the mean values of each group.
[b]TBA-reactive substances calculated as MDA equivalents.

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Cholesteryl Esterase Activities in Ventricles, Isolated Heart Cells and Aorta of the Rat

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Cholesteryl esterase activities were determined in homogenates of rat heart (ventricles), isolated, calcium-tolerant, cardiac myocytes and aortic tissue and were compared with acid and neutral triglyceride lipase activities in these fractions.

Using cholesteryl oleate/phosphatidylcholine/taurocholate emulsions and digitonin pretreatment of the enzyme fractions, acid and neutral cholesteryl esterase activities were measured in all tissue preparations. In contrast to the acid and neutral triglyceridase and acid cholesteryl esterase activity, the neutral cholesteryl esterase activity was subject to substrate inhibition. Upon isolation of cardiac myocytes, and in contrast with the recovery of neutral triglyceride lipase activity, only a small portion of the neutral cholesteryl esterase (6%) was recovered, suggesting that nonmyocyte neutral cholesteryl esterase activity markedly contributes to the relatively high activity detectable in whole ventricular homogenates. The recovery of large amounts of neutral cholesteryl esterase activity in the supernatant of collagenase-digested heart tissue, obtained during the isolation of myocytes, which is also markedly enriched in activities of two endothelial marker enzymes (5′-nucleotidase and angiotensine-converting enzyme) may indicate the predominant contribution of neutral cholesteryl esterase activity from coronary endothelial cells to this activity detectable in ventricular homogenates. Relative to the activity in ventricular and myocyte homogenates, aorta homogenates possessed the highest specific neutral cholesteryl esterase activity. We propose that in addition to coronary endothelium, smooth muscle cells also contribute to the neutral cholesteryl esterase activity in ventricular homogenates. Pretreatment of rats with carrageenan, an agent toxic to macrophages, lymphocytes and fibroblasts, induced a significant drop in myocardial neutral cholesteryl esterase and triglyceride lipase activity, suggesting that interstitially trapped macrophages may also contribute to lipolytic activities present in whole ventricular homogenates. Our data indicate that caution has to be taken upon extrapolation of experimental findings in heart homogenates to myocardial muscle cells.
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In rat heart homogenates, the presence of various lipolytic activities involved in the hydrolysis of exogenous and endogenous triglycerides and cholesteryl esters has been known for a long time (1–8). However, one cannot conclude from the presence of lipolytic activities in tissue homogenates that these enzymes are present in the cardiac myocytes, since these cells comprise only 12–20% of the total amount of heart tissue cells, although they represent the bulk of the tissue by mass (9). In addition to the myocytes, the heart contains cells of vascular origin (endothelial and smooth muscle cells), connective tissue cells and cardioadipocytes but probably also (occasional) interstitially trapped fibroblasts, lymphocytes, macrophages and neutrophils that have infiltrated the heart tissue. High acid and neutral triglyceride lipase and cholesteryl esterase activities have been described for aortic tissue and cultured macrophages, fibroblasts, lymphocytes and neutrophils (8,10–19). Furthermore, neutral triglyceride lipase activities, including lipoprotein lipase, have been described in isolated rat heart myocytes (20–23).

Recently, Goldberg and Khoo demonstrated for the first time the presence of a neutral cholesteryl esterase activity in mouse and rat heart homogenates and isolated rat heart cells (24). The present study was undertaken to reevaluate the cholesteryl esterase activities in rat heart (ventricular) homogenates, isolated rat heart myocytes and rat aortic tissue. The occurrence of cholesteryl esterase activities in the tissue preparations was compared with the presence of intracellular triglyceride lipase activities. In addition, the contribution of cholesteryl esterase activities present in interstitially occurring cells to overall heart homogenate activities was estimated after pretreatment of the rats with carrageenan, a polygalactan toxic for macrophages, lymphocytes and fibroblasts (25,26).

MATERIALS AND METHODS

Animals. Male Wistar rats of 200–250 g were used throughout the study. They had free access to control laboratory chow and water and were kept under an artificial light cycle of 12 hr (07.00–19.00 hr). Carrageenan (25 mg/kg body weight, suspended in sterile 0.9% [w/v] NaCl) was injected intraperitoneally under light ether anesthesia. Data obtained from the carrageenan-treated rats were compared with rats only receiving saline. The animals were killed 24 hr after carrageenan treatment. Carrageenan and saline injections did not affect the food consumption of the animals.

Perfusion protocol. Under light ether anesthesia the hearts were removed quickly from the thorax and immersed in ice-cold perfusion buffer. Subsequently, they were arranged for a non-recirculating Langendorff perfusion with a modified Tyrode buffer containing 11.1 mM glucose as described previously (5). Nonventricular tissue was removed as much as possible, and the hearts were electrically stimulated at a rate of 300 beats/min. After a 10-min preperfusion, heparin (5 U/ml) was included in the buffer to remove vascular lipoprotein lipase. Heparin perfusion continued for 30 min. No lipase activity could be detected in the effluent thereafter. Finally, a 5-min heparin washout perfusion was done. After perfusion, the hearts (ventricles) were minced finely, homogenized in a 1-mM phosphate buffer (pH 7.4) and, when indicated, a low speed supernatant prepared as described previously (5).

Preparation of isolated, calcium-tolerant cardiac myocytes. Myocytes were prepared from hearts of fed rats using a modification of the method described by Farmer et al. (27). We used a Tyrode buffer ($[Ca^{2+}]$ = 1.35 mM) instead of a Krebs-Henseleit buffer ($[Ca^{2+}]$ =

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2.5 mM). In addition, we included 0.01% (w/v) butylated hydroxytoluene, an antioxidant, during all incubations and washings. Briefly, after preperfusion with Tyrode buffer (step 1), a 5-min Ca^{2+}-free perfusion took place (step 2) followed by a 5-min perfusion with buffer containing 50 μM Ca^{2+}, 0.1% (w/v) collagenase, 0.1% (w/v) hyaluronidase and 0.1% (w/v) bovine serum albumin at a flow rate of 8 ml/min (step 3). Then the hearts were removed from the apparatus, two vertical slashes were made toward the apex of the heart and the tissue was incubated at 37 C with buffer supplemented with 50 μM Ca^{2+}, 0.1% (w/v) collagenase and hyaluronidase and 2% (w/v) bovine serum albumin (step 4). Then the disaggregated cells were collected by sieving through a 300-μ nylon mesh. The first cell suspension was discarded. After step 4 three successive 5-min incubations with the same medium were done followed by sieving (step 5). Thereafter, when almost the whole tissue was digested, the cells were collected by centrifugation (40 $\times$ g, 1 min) and washed three times with Tyrode buffer containing 50 μM Ca^{2+} and 2% (w/v) bovine serum albumin (step 6). The final cell pellet was suspended in control Tyrode buffer for characterization. In our lab, this method gives a 41.7 $\pm$ 0.58% (n = 8) cell yield on protein basis with a viability, as determined from 0.3% (w/v) trypan blue exclusion, of 70–80%. Sixty to 75% of the cells were rod-shaped; 10–20% of them were beating spontaneously. The myocytes were finally centrifuged, and the pellet was homogenized in a 1-mM phosphate buffer (pH 7.4) (step 7). When indicated, a low speed supernatant was prepared as described previously (5).

Aorta preparation. After removal of the heart for perfusion, the thoracic aorta was dissected free and stripped from nonarterial tissue. Aortas were washed thoroughly free of blood components using 0.9% (w/v) NaCl. All handlings took place in the cold room (4 C). Aortic tissue was finely minced, homogenized in a 1-mM phosphate buffer (pH 7.4) by two 30-second bursts of a Polytron tissue homogenizer (setting 5) and, when indicated, a low speed supernatant was prepared by centrifugation (5 min, 300 $\times$ g).

Determination of cholesteryl esterase activities (EC 3.1.1.13). Cholesteryl esterase activities in the various enzyme sources (homogenates or low speed supernatants) were initially tested using three different cholesteryl [1-^{14}C]oleate preparations. We used cholesteryl oleate/phosphatidylcholine/glycerol dispersions as described by Severson and Fletcher (8) (assay A), cholesteryl oleate/phosphatidylcholine/bile salt micelles according to Hajjar et al. (15) (assay B) and cholesteryl oleate/phosphatidylcholine/bile salt micelles after digitonin pretreatment of the enzyme preparations (0.025 mg/ml, 10 min, 4 C) according to Haley et al. (12) (assay C). All incubations were performed at 37 C for 30 min.

Assay A, final volume 100 μl, contained 50 μM [^{14}C]cholesteryl oleate (186 Becq/nmol), 320 μM phosphatidylcholine, 0.4% (w/v) bovine serum albumin (fatty acid-free), 1.25 M glycerol and 50 mM Tris-HCl/acetate buffer (pH range 3–9).

Assay B, final volume 125 μl, contained 50 μM [^{14}C]cholesteryl oleate (238 Becq/nmol), 200 μM phosphatidylcholine, 0.04% (w/v) bovine serum albumin, 100 μM sodium taurocholate and 100 mM potassium phosphate/acetate buffer (pH range 3–9).

Assay C, final volume 125 μl, contained 50 μM [^{14}C]cholesteryl oleate (359 Becq/nmol), 4.35 mM phosphatidylcholine, 2.0 mM sodium taurocholate and 125 mM Tris-HCl/acetate buffer (pH range 3–9).

Extraction of the liberated labeled fatty acids occurred as described elsewhere (8,12,15).

Cholesteryl esterase activity is expressed as nmol fatty acid released from the cholesteryl oleate substrate per hr per mg protein. The interassay variation of both cholesteryl esterase activities was less than 2%.

Determination of triglyceride lipase activities (EC 3.1.1.3). Triglyceride lipase activities in the various homogenates or low speed supernatants were determined at pH 4.8, 7.4 and 8.2, using a [^{3}H]triolein/gum acacia suspension as previously described (5). Neutral (or alkaline) triglyceride lipase activity was estimated in the absence and presence of 15% (v/v) rat serum, obtained from rats fasted overnight and preheated for 60 min at 56 C to remove its lipolytic activity. Triglyceride lipase activity is expressed as nmol fatty acid released from the triolein substrate per min per mg protein.

Determination of 5′-nucleotidase (EC 3.1.2.5) and angiotensine-converting enzyme activities (EC 3.4.15.1). The activities of 5′-nucleotidase and angiotensine-converting enzyme, marker enzymes for endothelial cells (28,29), were determined as described previously (29).

Immunotitration. Polyclonal antibodies against rat heart heparin-releasable lipoprotein lipase were obtained from female goats as described earlier (5,30). Twenty-five μl of the antibody preparation inhibited 10 mU of lipoprotein lipase activity. Triglyceride lipase and cholesteryl esterase activity in the various enzyme preparations were incubated with control and anti-lipoprotein lipase γ-globulins (90 min, 4 C). Thereafter the assay was started by the addition of the substrate.

Chemical assays. Protein content of ventricular, aorta and myocyte preparations was determined according to Lowry et al. (31) using bovine serum albumin as standard. Total cholesteryl ester contents of the tissue and cell preparations were determined, after organic extraction according to Bligh and Dyer (32) and drying, using a Boehringer test combination, based on methods described by Stähler et al. (31) and Trindler (32).

Reagents. Reagents and chemicals (all of analytical grade) were obtained from Merck (Darmstadt, Federal Republic of Germany) and Boehringer (Mannheim, Federal Republic of Germany). Bovine serum albumin (fraction V), collagenase (type I) and hyaluronidase (type II) were purchased from Sigma (St. Louis, Missouri). The albumin was freed from fatty acids by charcoal treatment. [9,10(n)-^{3}H]Glycerol triolein and cholesteryl [1-^{14}C]oleate were from Amersham International PLC (Amersham, United Kingdom), Instagel was from Packard (Downers Grove, Illinois) and heparin was from Organon (Oss, The Netherlands). Carrageenan was from Sigma and a gift from E. J. Schenkelaars from the Department of Pharmacology of our faculty.

Statistics. Most data are presented as mean values ($\overline{X}$) $\pm$ standard error of the mean (SEM), with n representing the number of experiments. Statistical significance of differences was calculated with Student's t-test (two-tailed). $P > 0.05$ was considered not significant (NS).

RESULTS

Validation of assays. The triolein, emulsified in gum acacia, is well established as a suitable substrate for the

assay of lipase activities in various tissue homogenates, and the activity of acid and neutral triglyceride lipase and lipoprotein lipase in heart tissue can be compared with data obtained with glycerol/triolein dispersions, ethanolic triolein substrates, [^{3}H]triolein-labeled chylomicrons and triolein/phosphatidylcholine micelles (3,35, 36). As demonstrated previously for low speed supernatants of whole heart homogenates (5), the triglyceride lipase activities in myocyte and aorta preparations also exhibited classical Michaelis-Menten kinetics (not shown). In addition, the lipase activities of all preparations were linear with time and increasing amounts of enzyme protein (not shown).

Previous work of Hajjar et al. (15) has indicated that the physical dispersion of cholesteryl oleate largely determines the actual rate of its hydrolysis by cholesteryl esterase. Figure 1 illustrates the pH dependence of cholesteryl esterase activity in low speed supernatants of ventricular homogenates using three different substrate preparations varying in their molar ratio of cholesteryl oleate, phosphatidylcholine and sodium taurocholate, preincubation of the enzyme fraction with digitonin, the stabilizing presence of glycerol and the presence of bovine serum albumin (assays A, B and C; see Materials and Methods). In all three assays, cholesteryl esterase activity exhibited two pH peaks, of which the actual rates and optima were different (see below). The highest acid cholesteryl esterase activity was measured using the glycerol-dispersed cholesteryl oleate/phosphatidylcholine micelles (assay A). However, using this preparation, only a low neutral cholesteryl esterase activity peak could be detected. By contrast, the use of sodium taurocholate yielded lower acid but higher neutral cholesteryl esterase activities. The substrate preparation described by Haley et al. (12) (assay C), using digitonin preincubation of the low speed supernatant, revealed a higher acid and neutral cholesteryl esterase activity compared with the substrate described by Hajjar et al. (15) applying cholesteryl oleate, phosphatidylcholine and taurocholate in a different ratio. Since this was also the case in low speed supernatants of aorta and myocyte homogenates, assay C was chosen for routine cholesteryl esterase determinations. As illustrated in Figure 2, the acid and neutral cholesteryl esterase activity of all enzyme preparations was linear with time for at least 40 min of incubation. Thereafter linearity was lost. During routine cholesteryl esterase assays we used an incubation period of 30 min. In addition, as presented in Figure 3, the acid and neutral cholesteryl esterase

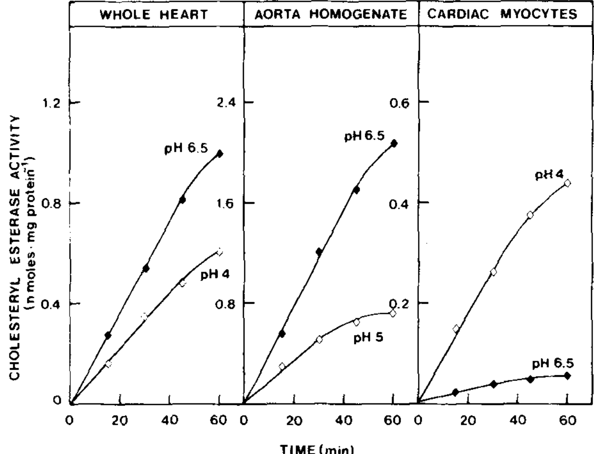

FIG. 2. Time dependency of acid and neutral cholesteryl esterase activity in low speed supernatants of homogenates prepared from whole rat heart (ventricles), aorta and isolated cardiac myocytes. For further details see Fig. 1 and Materials and Methods. Results are the mean values of two separate experiments.

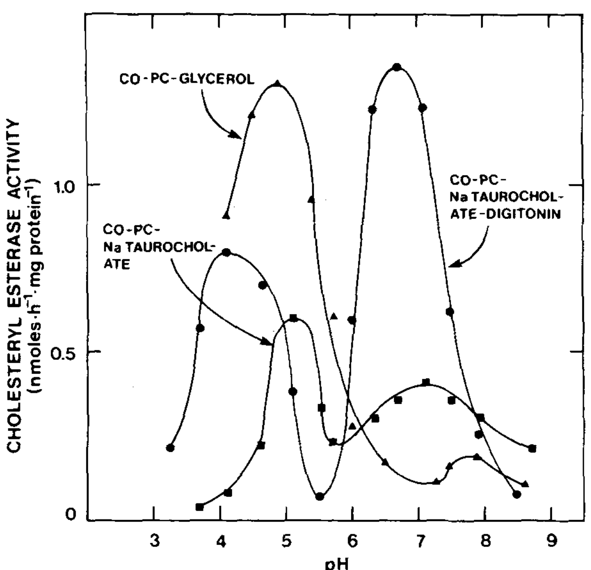

FIG. 1. The effect of substrate preparation on pH dependence of cholesteryl esterase activity in low speed supernatants of rat heart (ventricular) homogenates. Cholesteryl oleate (CO) substrate emulsions were prepared as described in Materials and Methods. The preparations were ▲: 50 μM CO, 320 μM phosphatidylcholine (PC), 1.25 M glycerol; □: 50 μM CO, 200 μM PC, 100 μM taurocholate; ●: 50 μM CO, 4.35 mM PC, 2.0 mM taurocholate after digitonin pretreatment of the enzyme fraction (0.025 mg/ml, 10 min at 4 C). The ● assay was used in routine determinations of cholesteryl esterase activity. The results are the mean value of two separate experiments.

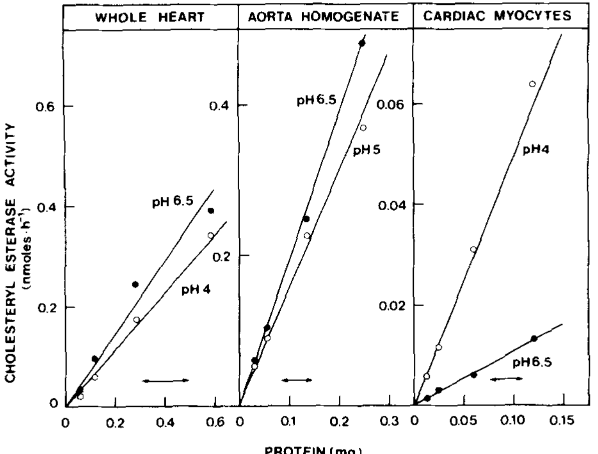

FIG. 3. Protein dependency of acid and neutral cholesteryl esterase activity in low speed supernatants of homogenates prepared from whole rat heart (ventricles), aorta and isolated cardiac myocytes. For further details see Fig. 1 and Materials and Methods. Results are the mean values of two separate experiments. The horizontal bars indicate the amount of protein in the low speed supernatant used during routine cholesteryl esterase assays.

activity of all tissue preparations was linear with increasing amounts of enzyme protein. The neutral cholesteryl esterase activity (pH 6.5; see below) in low speed supernatants of ventricular, aorta and myocyte homogenates was subject to substrate inhibition at cholesteryl oleate concentrations above 50 μM (Fig. 4). Acid cholesteryl esterase activity, in contrast, did not show this phenomenon in low speed supernatants of heart homogenates (activity peak at pH 4) and was only moderately inhibited at higher cholesteryl oleate concentrations in aortic and myocyte preparations (activity peak at pH 5 and 4, respectively).

Based upon the total endogenous cholesterol content of the used tissue preparations (7.3 μg/mg protein in ventricles, 33.2 in aorta and 6.3 in the myocyte preparations, of which less than 5% was in esterified form; $\bar{X}$, n = 2), we calculated that endogenous cholesteryl esters maximally enhanced the assay concentration by about 0.5%. Together with the protein linearity of the assay, we conclude that endogenous cholesteryl esters were not hydrolyzed in preference nor did they dilute the radioactivity of the added substrate significantly. The described substrate inhibition can therefore not be attributed to effects of endogenous cholesteryl esters.

Acid and neutral cholesteryl esterase activities in ventricular tissue, aorta and isolated myocytes compared with triglyceride lipase activities. As presented in Figure 5, the low speed supernatant fractions of whole ventricular, aortic and myocyte homogenates contain acid (pH 4.0, 5.0 and 4.2, respectively) and neutral (pH 6.5) cholesteryl esterase activity peaks. Aortic cholesteryl esterase activities were the highest on a mg protein basis. Strikingly, the neutral cholesteryl esterase activity in low speed supernatants of whole ventricular homogenates was recovered to only a small extent in the myocyte preparation. This difference in specific activity may indicate that the bulk of the heart enzyme was not associated with the myocytes but with other cell types present in the myocardial tissue.

The pH activity profiles of cholesteryl esterase in ventricular, aortic and myocyte preparations were compared

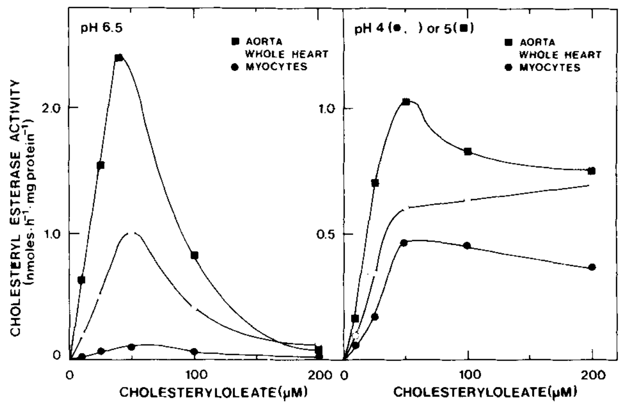

FIG. 4. The effect of increasing cholesteryl oleate concentrations on the activity of acid and neutral cholesteryl esterase in low speed supernatants of homogenates prepared from rat heart (ventricles) (O), aorta (■) and isolated cardiac myocytes (●). For further details see legend to Fig. 1 and Materials and Methods. Results are the mean values of two separate experiments.

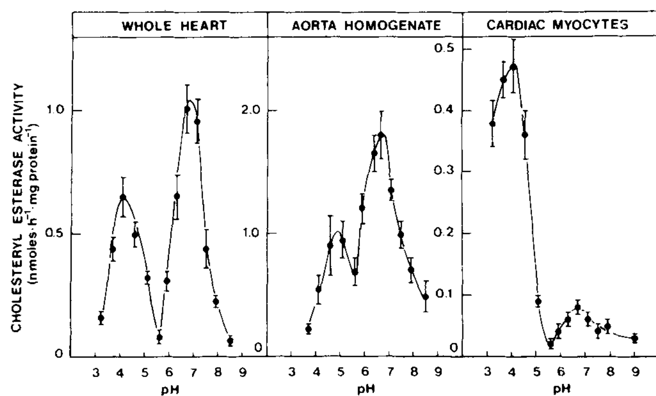

FIG. 5. pH dependence of cholesteryl esterase activities in low speed supernatants of homogenates from rat heart (ventricles), aorta and isolated cardiac myocytes. For further details see legend to Fig. 1 and Materials and Methods. Results are the mean values ± SEM of 4–8 experiments.

with those of the acid and neutral (or alkaline) triglyceride lipase activities. The low speed supernatants of ventricular, aorta and myocyte homogenates contain acid triglyceride lipase activity peaks of pH 4.8, 5.5 and 5, respectively. The ventricular neutral triglyceride lipase activity, determined in the absence of serum, peaked at pH 7.4. This activity was markedly stimulated during incubation in the presence of serum. In the aortic and myocyte preparation, the pH peak of serum-activated triglyceride lipase activity shifted to alkaline regions (pH 8.2).

In contrast to the basal and serum-stimulated neutral triglyceridase activity (5), the neutral cholesteryl esterase activity of all three preparations was not inhibited by preincubation with anti-lipoprotein lipase γ-globulins (not shown).

Two successive washings of the final myocyte preparation with heparin (5 U/ml) induced only a 11 ± 2% (n = 3) reduction of basal and serum-stimulated neutral triglyceride lipase activity and did not affect the neutral cholesteryl esterase activity. This suggests a predominant intracellular origin of the neutral triglyceridase activity in myocytes.

Table 1 summarizes the absolute recoveries of protein and lipolytic activities during the isolation of myocytes from rat hearts. Protein recovery in myocyte homogenates amounted to about 40%. Myocyte homogenate acid and neutral triglyceride lipase and acid cholesteryl esterase activities were recovered by about 30% relative to their activity in whole heart homogenates. The 10% difference may be a consequence of the presence of nonviable dead cells in our myocyte preparations (20–30%). Of the neutral cholesteryl esterase activity detectable in whole ventricular homogenates, only about 6% was recovered after isolation of the myocytes. The loss of neutral cholesteryl esterase activity upon myocyte isolation indeed suggests that its activity in homogenates is associated with other nonmyocyte cell types in the heart.

Acid and neutral cholesteryl esterase activity in fractions obtained during the isolation of calcium-tolerant myocytes from adult rat hearts. The low recovery of neutral cholesteryl esterase activity in the final myocyte fraction (Table 1) prompted us to study its recovery in other fractions obtained during the isolation of myocytes

TABLE 1

Recovery of Protein and Lipolytic Enzyme Activities After the Isolation of Calcium-Tolerant Myocytes from Adult Rat Hearts

	Ventricular homogenate	Myocyte homogenate
Total protein (mg)	198.3 ± 5.4 (100%)	82.7 ± 1.1 (41.7 ± 0.5%)
Acid triglyceride lipase (nmol/min)	42.1 ± 1.6 (100%)	7.1 ± 0.3 (16.9 ± 1.3%)
Neutral triglyceride lipase − serum (nmol/min)	23.9 ± 2.6 (100%)	6.3 ± 0.2 (26.4 ± 1.0%)
Neutral triglyceride lipase + serum (15% v/v) (nmol/min)	199.8 ± 9.4 (100%)	58.4 ± 0.6 (29.2 ± 0.3%)
Acid cholesteryl esteras (nmol/hr)	103.2 ± 1.7 (100%)	31.9 ± 1.3 (30.9 ± 1.3%)
Neutral cholesteryl esterase (nmol/hr)	126.5 ± 6.5 (100%)	7.2 ± 0.3 (5.7 ± 0.2%)

Adult rat hearts were preperfused with heparin and subsequently homogenized in a 1-mM phosphate buffer of pH 7.4. Cardiac myocytes were isolated from adult rat hearts and the final pellet was homogenized as above. Total protein content and lipolytic activities in ventricular and myocyte homogenates were measured. Protein content and lipolytic activities in ventricular homogenates were taken as 100%. Values obtained in the myocyte homogenate were expressed as percentages of values determined in the ventricular homogenates (see parentheses). Results represent the mean values ± SEM of 4-8 separate experiments. For further details, see Materials and Methods.

from adult rat hearts. The following fractions were collected and investigated for total activities of acid and neutral cholesteryl esterase and two endothelial enzyme markers, 5′-nucleotidase and angiotensine-converting enzyme (28,29): a) the myocardial effluent collected during collagenase- and hyaluronidase perfusion (step 3); b) the cell suspension from the first incubation of preperfused heart tissue with collagenase and hyaluronidase (step 4); c) the final supernatant of completely digested tissue after pelleting the myocytes by centrifugation (step 5); d) the combined supernatants obtained after the washing of the myocytes (step 6); and e) the final myocyte preparation. The percent distribution of cholesteryl esterases and markers between the collected fractions is presented in Table 2. Overall recovery of enzyme activities in the isolated fractions, compared with the total activity in whole heart homogenates, was 126.3% for the acid cholesteryl esterase, 94.7% for the neutral cholesteryl esterase, 104.3% for the 5′-nucleotidase and 107.3% for the angiotensine-converting enzyme.

From Table 2 we conclude that, in contrast to the acid cholesteryl esterase activity, a large part of the neutral cholesteryl esterase was recovered in the supernatant fraction obtained from the digested heart tissue by centrifugation (step 5). This fraction was also markedly enriched in both endothelial cell enzyme markers. Acid cholesteryl esterase activity was recovered predominantly in the final myocyte pellet. The endothelial contamination of this fraction is relatively small: from the recovery of the marker enzymes we estimate about 3-11%. In contrast to 5′-nucleotidase, the angiotensine-converting enzyme was also recovered in substantial amounts in the effluent collected during collagenase and hyaluronidase perfusion (step 3). This "release" may be a consequence of the loose binding of the enzyme to the endothelial cell membrane (37) and does not reflect the washout of endothelial cells, since the 5′-nucleotidase release was low.

Effect of carrageenan pretreatment of rats on myocardial cholesteryl esterase and triglyceride lipase activities. To investigate the contribution of lipolytic activites from other cell types (macrophages, lymphocytes and fibroblasts) to activities detectable in whole ventricular homogenates, we treated rats with carrageenan (intraperitoneally administered) and determined the lipolytic activity in whole ventricular homogenates 24 hr later. Carrageenan is proposed to possess a selective toxicity for macrophages and other (occasionally interstitial) cell types (fibroblasts, lymphocytes) throughout the body (25, 26). The dose of the intraperitoneally injected carrageenan (25 mg/kg body weight) and the duration of the treatment (24 hr) were chosen based on data from Fowler and Thomson (28). As demonstrated in Table 3, carrageenan treatment induced a small but statistically significant decrease in neutral triglyceride lipase and predominantly the neutral cholesteryl esterase activity. The small reduction in the activity of lysosomal lipolytic enzymes was not statistically significant. Since the interassay variation was much lower than the standard deviation of lipase and esterase activity in control and carrageenan-treated rats, the significant drop in both neutral enzymes induced by carrageenan truly reflects an effect of this drug.

DISCUSSION

Although isolated perfused organs and their homogenate or subcellular fractions are frequently used in metabolic studies, one must be aware that tissues are heterogenous and it may be difficult to extrapolate findings in fractionated tissue to the main cell type in such a tissue.

The presence of different metabolic tissue compartments in perfused rat hearts was demonstrated by us previously using a modified perfusion technique allowing the separate collection of interstitial fluid and coronary vascular effluent (39,40). The composition of both effluents was completely different, revealing that metabolites and enzymes from cardiac myocytes (glycerol, lactate, fatty acids, proteins, lactate dehydrogenase) were mainly recovered in the interstitial fluid, whereas products from vascular origin (prostaglandin E_2 and I_2, lysophosphoglycerides, lipid peroxidation products, lipoprotein lipase) were largely recovered in the coronary venous effluent.

In the present study, we have described and partially characterized cholesteryl esterase activities in low speed supernatants of homogenates of rat hearts, isolated cardiac myocytes and aortic tissue.

Our experiments again point out the importance of the mode of substrate preparation in the cholesteryl esterase assay (12-14). Using the preparation described by Haley et al. (12), acid and neutral cholesteryl esterase activities in all enzyme fractions could easily be detected. Using glycerol dispersions of cholesteryl oleate, a lower neutral cholesteryl esterase activity was detected. All tissue

TABLE 2

Distribution of Acid and Neutral Cholesteryl Esterase Activities and the Endothelial Marker Enzymes 5′-Nucleotidase and Angiotensine-Converting Enzyme Between Fraction Obtained During the Stepwise Isolation of Cardiac Myocytes from Adult Rat Hearts

Fraction (isolation step)	% Distribution of activity			
	Acid cholesteryl esterase	Neutral cholesteryl esterase	5′-Nucleotidase	Angiotensine-converting enzyme
Effluent from collagenase + hyaluronidase perfused hearts (step 3)	7.5 ± 1.3	10.3 ± 1.9	5.2 ± 2.2	29.5 ± 2.7
Discarded cells from the collagenase + hyaluronidase incubation of heart tissue (step 4)	3.9 ± 0.1	9.3 ± 1.6	5.3 ± 0.4	9.5 ± 0.2
Total supernatant of the digested heart tissue (step 5)	25.4 ± 1.2	52.5 ± 1.7	53.6 ± 2.4	46.1 ± 2.1
Total myocyte-washing supernatant (step 6)	21.4 ± 1.9	21.7 ± 0.3	22.4 ± 0.7	11.5 ± 0.7
Final myocyte preparation	41.8 ± 1.8	6.2 ± 0.4	13.5 ± 1.2	3.4 ± 0.3

In fractions obtained during the isolation of cardiac myocytes from adult rat hearts, total acid and neutral cholesteryl esterase, 5′-nucleotidase and angiotensine-converting enzyme activities were determined. The sum of the activities was taken as 100%. Results represent the mean values ± SEM of 4 separate experiments. For further details, see Materials and Methods.

TABLE 3

Effect of Carrageenan Pretreatment of Rats on Ventricular Cholesteryl Esterase and Triglyceride Lipase Activities

Enzyme	pH	Control	Carrageenan
Cholesteryl esterase (nmol/hr/mg)	4.0	0.66 ± 0.02 (13)	0.58 ± 0.11 (4)
Cholesteryl esterase (nmol/hr/mg)	6.5	1.02 ± 0.06 (13)	0.85 ± 0.05 (4)[a]
Triglyceride lipase (nmol/min/mg)	4.8	0.53 ± 0.04 (6)	0.45 ± 0.02 (4)
Triglyceride lipase (−) (nmol/min/mg)	7.4	0.56 ± 0.08 (6)	0.45 ± 0.03 (4)
Triglyceride lipase (+) (nmol/min/mg)	7.4	1.92 ± 0.05 (6)	1.76 ± 0.03 (4)[a]

Rats were intraperitoneally injected with carrageenan (25 mg/kg body weight) suspended in sterile 0.9% (w/v) NaCl. Control animals only received saline. Hearts were perfused with heparin (5 U/ml) 24 hr later to remove vascular lipoprotein lipase. Thereafter, the ventricles were homogenized and a low speed supernatant was prepared by centrifugation. Acid and neutral triglyceride lipase and cholesteryl esterase activities were measured at the indicated pH. Neutral triglyceride lipase activity was determined in the absence (−) and presence (+) of 15% (v/v) rat serum. The data are presented in mean values ± SEM. The number of experiments is given in parentheses. For further details, see Materials and Methods.

[a]$p < 0.05$ vs the control group.

preparations contained an acid cholesteryl esterase activity, probably from lysosomal origin. Our data with respect to the neutral cholesteryl esterase in rat heart homogenates confirms recent findings of Goldberg and Khoo (24) who, for the first time, described this neutral activity in mouse heart postmitochondrial fractions. The neutral cholesteryl esterase in rat heart homogenates, myocytes and aorta was subject to substrate inhibition. This phenomenon excludes meaningful application of conventional kinetic modeling and analysis. Substrate inhibition of neutral cholesteryl esterase activity was also observed in rat testis (41).

Aortic neutral cholesteryl esterase activity is probably associated with the vascular smooth muscle cells and involved in the mobilization of stored cytosolic cholesteryl esters (42). An intracellular localization is therefore conceivable. Upon isolation of myocytes from rat heart, a large part of the neutral cholesteryl esterase was lost. Only about 6% of total rat heart homogenate activity was recovered, whereas the acid cholesteryl esterase was recovered for 30.9%.

All enzyme preparations contained an acid triglyceride lipase, presumably of lysosomal origin (3,29). In accordance with earlier findings also, a neutral triglyceride lipase activity was present in all preparations. This lipase exhibited all characteristics of lipoprotein lipase (stimulation by serum, inhibition by 1 M NaCl and almost complete inhibition by anti-lipoprotein lipase antibodies). The presence of lipoprotein lipase in isolated myocytes and aorta confirms previous results (19–27,43). In addition, Ramirez et al. (21) and Goldberg and Khoo (24) have recently described a very low and labile activity of a

neutral lipase apparently distinct from lipoprotein lipase in isolated rat myocytes.

The recovery of the neutral triglyceride lipase and neutral cholesteryl esterase, both presumably intracellular enzymes, during the preparation of isolated myocytes was strikingly different (30% vs 6%, respectively). The overall recovery of acid triglyceride lipase, the acid cholesteryl esterase and neutral triglyceride lipase activity were 16.9, 30.9 and 26.4%, respectively. The rather dramatic loss of neutral cholesteryl esterase activity during the isolation of myocytes may be explained by a) a selective inactivation of this enzyme during the isolation procedure or b) its predominant localization in nonmyocardial cardiac tissue cells or interstitially occurring macrophages, lymphocytes and fibroblasts. There are no arguments at hand for a selective inactivation of the neutral cholesteryl esterase. The overall recovery of neutral cholesteryl esterase activity in fractions obtained during the isolation of myocytes was 95% compared with whole heart homogenates. This excludes the presence of an activator in whole tissue homogenates that is lost during myocyte isolation or selective inactivation of the enzyme in the myocyte fraction. Moreover, the 30% recovery of the rather labile intracellular neutral triglyceridase followed the overall recovery of viable cells on a protein basis.

A contribution of neutral cholesteryl esterase from other cell types in heart tissue is therefore more likely. The concomitant recovery of the bulk of the neutral cholesteryl esterase activity and the marker enzymes for endothelial cells (5′-nucleotidase and angiotensine-converting enzyme) in the supernatant of collagenase-treated heart tissue (step 4) indicated that coronary vascular endothelium may substantially contribute to the neutral cholesteryl esterase activity detectable in ventricular homogenates. Assuming that aortic tissue is a reliable marker for overall (coronary) vascular tissue, it is tempting to speculate on the additional contribution of neutral cholesteryl esterase activity from vascular smooth muscle cells to ventricular homogenates, especially since the aortic cholesteryl esterase has the highest specific activity compared to ventricular homogenates and isolated myocytes. In addition, our experiments using carrageenan, a sulfated polygalactose that is very toxic to macrophages, fibroblasts and lymphocytes, indicate that also neutral cholesteryl esterase and triglyceride lipase activities present in these carrageenan-sensitive cells contribute to overall ventricular homogenate activities. Unfortunately, there is no reliable enzymatic marker for the above cells, so it is not possible to evaluate the actual carrageenan action. Finally, myocardial adipocytes may contribute to all lipolytic activities in tissue homogenates, but this contribution is difficult to estimate in the experimental set-up used.

In conclusion, our experiments indicate the presence of acid and neutral cholesteryl esterase activity in rat ventricular homogenates, aortic tissue and isolated cardiac myocytes. The relatively high activity of neutral cholesteryl esterase in ventricular homogenates may largely be determined by activities from nonmyocyte tissue cells in the heart. We propose that cells from vascular origin, in particular endothelial cells, and interstitially occurring carrageenan-sensitive cells are good candidates. Extrapolation of enzymic findings in whole heart (or ventricular) homogenates to processes in cardiac myocytes may therefore easily lead to misinterpretation.

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Cecile Hanson and Martha Wieriks typed the manuscript.

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Resolution of Radiolabeled Molecular Species of Phospholipid in Human Platelets: Effect of Thrombin

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Resolution of individual molecular species of human platelet 1,2-diradyl-*sn*-glycero-3-phosphocholines and 1,2-diradyl-*sn*-glycero-3-phosphoethanolamines by reverse phase high pressure liquid chromatography (HPLC) allowed a thorough analysis of those phospholipids labeled with [^{3}H]arachidonic acid. Approximately 54% and 16% of the total incorporated radiolabel was found in choline glycerophospholipids and ethanolamine glycerophospholipids, respectively, with ca. 90% of this being found in the 1,2-diacyl molecular species. Eighty percent of [^{3}H]-arachidonic acid incorporated into 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine in resting platelets was equally distributed between 1-palmitoyl-2-arachidonoyl and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, while 70% of the radiolabel in 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine was found in 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine. Thrombin stimulation (5 U/ml for 5 min) resulted in deacylation of all 1-acyl-2-[^{3}H]arachidonoyl molecular species of 1-acyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine and 1-acyl-2-arachidonoyl-*sn*-glycero-3-ethanolamine. There was also a slight increase in 1-O-alkyl-2-[^{3}H]arachidonoyl-*sn*-glycero-3-phosphocholine and a significant increase in 1-O-alk-1′-enyl-2-[^{3}H]arachidonoyl-*sn*-glycero-3-phosphoethanolamine molecular species of over 300%. Thus, HPLC methodology indicates that arachidonoyl-containing molecular species of phosphatidylcholine and phosphatidylethanolamine are the major source of arachidonic acid in thrombin-stimulated human platelets, while certain ether phospholipid molecular species become enriched in arachidonate.
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Analysis of 1,2-diradyl-*sn*-glycero-3-phosphocholine of human platelets has shown that 81% of the phospholipid mass consists of 1,2-diacyl-*sn*-glycero-3-phosphocholine (1,2-diacyl GPC) molecular species, while two ether phospholipid subclasses, namely 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (8.8%) and 1-O-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine (9.2%), constituted the remainder (1). It also is known that over 60% of human platelet 1,2-diradyl-*sn*-glycero-3-phosphoethanolamine is 1-O-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine (i.e., plasmalogen) (1–3). Further analysis showed that only 3% of 1-radyl-2-acyl-*sn*-glycero-3-phosphoethanolamine was the 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine subclass, with the remainder having the 1,2-diacyl-*sn*-glycero-3-ethanolamine structure (1,2-diacyl GPE) (1). Finally, 1,2-diradyl-*sn*-glycero-3-phosphoinositol (1,2-diradyl GPI) has been shown to contain 1% 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphoinositol, with the rest being 1,2-diacyl-*sn*-glycero-3-phosphoinositol (1,2-diacyl GPI) (4).

Mahadevappa and Holub (5–7) have analyzed completely the diacyl molecular species of phospholipid from human platelets using snake venom phospholipase A_2 for release of *sn*-2 fatty acids and argentation thin layer chromatography; however, ether subclasses were not considered. High pressure liquid chromatography (HPLC) procedures are now available for separating a given phospholipid class into its individual molecular species of phospholipid (8). Studies by Kramer et al. (9) on the incorporation of radiolabeled 1-O-hexadecyl-2-lyso-*sn*-3-glycerophosphocholine into 1-O-hexadecyl-2-acyl-*sn*-3-glycerophosphocholine in human platelets utilized this methodology. This same approach had been shown to separate 1,2-diacyl, 1-O-alkyl-2-acyl and 1-O-alk-1′-enyl-2-acyl structural analogues of neutrophil phospholipid labeled with [^{3}H]arachidonic acid (10). Thus, we decided to analyze the individual molecular species of phospholipid radiolabeled with arachidonic acid in both unstimulated platelets and cells activated with thrombin to ascertain changes in individual molecular species of phospholipid.

MATERIALS AND METHODS

All chemicals were reagent grade or better. All solvents were HPLC grade (EM Science, Omnisolv) and were obtained from Bodman Chemicals (Media, Pennsylvania). Primulin dye was purchased from Sigma (St. Louis, Missouri). Silica Gel H thin layer chromatography (TLC) plates were obtained from Analtech (Newark, Delaware). Phospholipid standards were purchased from Serdary (London, Ontario, Canada) and Avanti (Birmingham, Alabama). Tritiated arachidonic acid (5,6,8,9,11,12,14,15,-^{3}H) was obtained from Amersham (Arlington Heights, Illinois) and had a specific activity of 137 Ci/mmol, while [^{14}C(U)]arachidonic acid (sp act 390 mCi/mmol) and [12,13-^{3}H]linoleic acid (sp act 49.0 Ci/mmol) were from New England Nuclear (Dupont, Boston, Massachusetts). Betafluor was a product of National Diagnostics (Somerville, New Jersey). The HPLC column was a 4.6 $\times$ 250 mm ultrasphere ODS (5 μm particle size) purchased from Beckman Instruments (Berkeley, California).

Isolation of human platelets. Blood was collected in 1/6 vol ACD (2.5 g trisodium citrate, 1.5 g citric acid, 2.0 g glucose/100 ml H_2O) solution and supernatant platelet-rich plasma (PRP) removed after centrifugation at 200 $\times$ g for 15 min at 22 C. Fifty ml PRP (pH 6.5) was centrifuged at 2600 $\times$ g for 15 min at 22 C, and the pellet was gently resuspended in 1/10 vol supernatant plasma. This was incubated with the appropriate radioactive fatty acid(s) (solvent evaporated) for 1 hr at 37 C. Labeling was done either with 50 μCi [^{3}H]arachidonic acid or for double labeling experiments 25 μCi [^{3}H]linoleic and 2 μCi [^{14}C]arachidonic. The PRP was allowed to cool to 22 C and gel was filtered on a Sepharose 2B column using Tyrodes buffer with an albumin concentration of 0.2% w/v as previously described (11). Platelet concentration was determined by Coulter counter (11) and then adjusted to 10^9 cells/ml.

Thrombin. Ten thousand units of thrombin (Parke-Davis, Detroit, Michigan) was dialyzed against 0.15 M

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NaCl for 12 hr to remove calcium, then diluted to a concentration of 100 units/ml and kept frozen.

Extraction and fractionation of phospholipids. Cellular lipids were extracted by the method of Bligh and Dyer (12). The choline- and ethanolamine-containing phospholipids were isolated by TLC on Silica Gel H plates using the solvent system of Skipski et al. (13). To determine the [^{3}H]arachidonic acid content in 1,2-diacyl-, 1-O-alkyl-2-acyl- and 1-O-alk-1′-enyl-2-acyl-, *sn*-glycero-3-phosphocholine (GPC) and *sn*-glycero-3-phosphoethanolamine (GPE), the TLC-resolved phospholipid classes of 1,2-diradyl-*sn*-glycero-3-phosphocholine and 1,2-diradyl-*sn*-glycero-3-phosphoethanolamine were treated for 12 hr with *Bacillus cereus* phospholipase C and acetylated as previously described (1,14). The 1,2-diradyl-glyceroacetates were resolved by TLC (14) and eluted from the silica gel, and the radioactivity was determined.

HPLC of intact phospholipid. The isocratic reverse phase system of Patton et al. (8), which uses 20 mM choline chloride in methanol/water/acetonitrile (90.5:7:2.5, v/v/v) as solvent at a flow rate of 1.0 ml/min, was used to resolve individual molecular species of a given class of phospholipid. This approach also resolves ether phospholipids from diacyl molecular species (10) and, for given structural analogues, the elution sequence is 1,2-diacyl, 1-O-alk-1′-enyl-2-acyl and 1-O-alkyl-2-acyl. The column was run at 37 C, and the sample was injected in a 1:1 solution of methylene chloride/methanol (10). Recovery of the applied phospholipid radioactivity was greater than 94%. In most experiments, the phospholipid from 3.3 × 10^8 cells for choline glycerophospholipids and 5 × 10^8 cells for ethanolamine glycerophospholipids was injected onto the column. To determine the elution of [^{3}H]arachidonate, 1-ml samples of eluate were collected and portions were counted to determine radioactivity. In other experiments, individual peaks were collected and total radioactivity was determined. Identification of individual peaks was done initially by comparison with the elution time of synthetic phospholipid standards and analysis of the fatty acid methyl esters of the phospholipid in individual peaks by gas liquid chromatography (GLC). 1-O-alkyl-2-arachidonoyl GPC standards were donated by R. Wykle (Department of Biochemistry, Bowman Gray School of Medicine, Winston-Salem, North Carolina). To verify identification, we used the procedures of this lab (1), Patton et al. (8), Mahedevappa and Holub (5), Kramer et al. (9) and Swendsen et al. (10). Generally, the Patton system gave some overlap of adjacent molecular species; however, most of the arachidonoyl-containing molecular species were well resolved. Phospholipid eluate was monitored at 206 nm.

GLC analysis of phospholipid. Eluate from the reverse phase column was evaporated to dryness and treated for 10 min with 2 M sodium methoxide at 50 C. The resulting fatty acid methyl esters were isolated and analyzed using a Hewlett-Packard chromatograph model 5730A as described elsewhere (11).

*Hydrolysis of 1-O-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine.* 1,2-Diradyl-*sn*-3-glycerophosphoethanolamine from platelets or material from individual eluate peaks obtained from HPLC was dissolved in chloroform/methanol (1:1, v/v) and dried so as to coat the inside of a 25-ml flask and then was exposed to HCl gas (1). The resulting 1-O-lyso-2-acyl-*sn*-3-glycero-phosphoethanolamine was separated from the intact 1,2-diacyl-*sn*-3-glycerophosphoethanolamine and 1-O-alkyl-2-acyl-*sn*-3-glycerophosphoethanolamine (only 3% of the total) by TLC (1). Subsequently, the lipid was eluted and compared with unhydrolyzed sample.

Following radioactive labeling and gel filtration, 1-ml samples (10^9 cells/ml) were incubated for 2 min at 37 C and time zero samples were taken. Coincidentally, thrombin was added to give a final concentration of 5 U/ml in duplicate samples for 5 min. Control samples were incubated in the absence of thrombin for the maximum time of the experiment. Samples were not stirred. Cells then were immediately added to chloroform/methanol (1:2, v/v) containing 0.01% butylated hydroxy toluene. This antioxidant also was present in all extraction and TLC solvents.

RESULTS

1,2-Diradyl GPC and GPE were isolated from cells incubated in the absence of thrombin for 5 min. In resting platelets, the [^{3}H]arachidonate radioactivity in 1-radyl-2-acyl GPC and GPE was 280,540 ± 22,580 cpm (mean ± SEM) and 81,522 ± 8,150 cpm per 10^9 cells (n = 6). This represented 54.3% and 15.8% of the total [^{3}H]arachidonate radiolabel incorporated into the cellular phospholipid. Analysis of diglyceride acetates gave the following contribution by subclasses to the total 1-radyl-2-acyl GPC: 1,2-diacyl, 93.4%; 1-alkyl-2-acyl, 5.6%; and 1-O-alk-1′-enyl-2-acyl, 1.0%. For 1-radyl-2-acyl GPE, the contribution for the same subclasses was 86.8%, 1.8% and 11.4%, respectively.

1,2-Diradyl GPC and GPE from resting human platelets radiolabeled with [^{3}H]arachidonic acid were subjected to reverse phase HPLC using the isocratic solvent system of Patton et al. (8). The elution profiles are shown in Figures 1 and 2. Figure legends describe identification of molecular species of phospholipid. The high amount of [^{3}H]arachidonate radioactivity in 1,2-diacyl GPC and GPE compared to ether lipid helped in identifying different molecular species. 1-O-alk-1′-enyl-2-acyl GPE molecular species (Fig. 2) were located by treatment of the plasmalogen with HCl gas (1).

The radioactivity in individual molecular species or separate peaks was determined for 1,2-diradyl GPC eluted from the reverse phase HPLC column. In control cells, radioactivity associated with peak 5 (18:2–20:4), peak 10 (16:0–20:4), peak 11 (18:1–20:4) and peak 20 (18:0–20:4) represented 5.9%, 8.9%, 39.4% and 37.7%, respectively, of the total radioactivity eluted from the column. As the relative contribution of 1,2-diacyl GPC to the total 1-radyl-2-acyl GPC radioactivity was known, the radioactivity contributed by a given molecular species could be determined. Such calculations were performed both for total 1-radyl-2-acyl GPC and GPE of control and thrombin-stimulated cells. Results for individual molecular species are shown in Table 1. In resting cells, 80% of the [^{3}H]arachidonate in 1,2-diacyl GPC was equally distributed between the molecular species 16:0–20:4 and 18:0–20:4 species, with the remainder being present in 18:1–20:4 and 18:2–20:4 GPC. Seventy percent of the [^{3}H]arachidonate in 1,2-diacyl GPE of resting cells was found in 18:0–20:4 GPE, with the remainder present in 18:1–20:4 and 16:0–20:4 GPE. Relatively little [^{3}H]arachidonate was observed in the three plasmalogen molecular

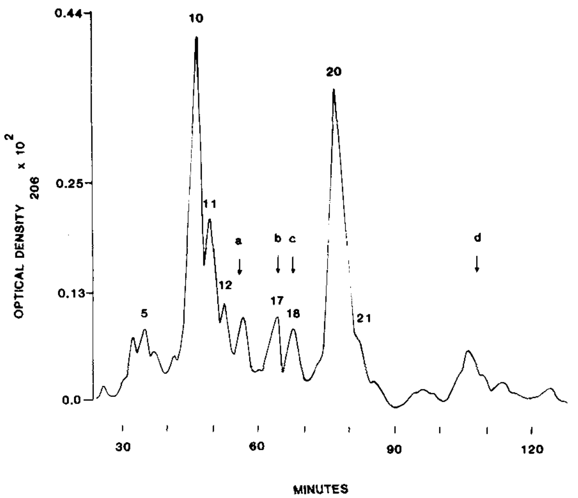

FIG. 1. Resolution of individual molecular species of 1,2-diradyl GPC by HPLC. The 1,2-diradyl GPC applied to the column was obtained from 3.3×10^8 resting platelets. Results in this and accompanying figures are representative of six separate experiments on different platelet preparations. 1,2-Diacyl molecular species are numbered according to Patton et al. (8). Letters over arrows indicate the elution position of known ether lipid standards determined by Swendsen et al. (10). Identification of 1,2-diacyl molecular species: peak 5, 18:2–20:4; 10, 16:0–20:4; 11, 16:0–18:2 (peak 11 is also coincident with 18:1–20:4, as shown in ref. 8); 12, 18:1–18:2; 17, 16:0–18:1; 18, 18:1–18:1; 20, 18:0–20:4; 21, 18:0–18:2. Identification of ether molecular species of 1,2-diradyl GPC: peak a, 16:0 alk-1′-enyl-20:4; b, 16:0 alkyl-20:4; c, 18:1 alkyl-20:4; d, 18:0 alkyl-20:4, as indicated by ref. 10.

TABLE 1

[^{3}H]Arachidonic Acid Individual Molecular Species of 1,2-Diradyl GPC and GPE in Control and Thrombin-Stimulated Human Platelets[a]

Molecular species	Control	Thrombin-stimulated
1,2-Diacyl PC		
18:2–20:4	16,770 ± 4,850	9,050 ± 3,900[b]
18:1–20:4	26,150 ± 2,510	13,430 ± 2,950[c]
16:0–20:4	114,100 ± 12,510	59,300 ± 7,820[c]
18:0–20:4	100,620 ± 13,150	53,700 ± 6,820[c]
1-O-alkyl-2-acyl GPC[d]	18,930 ± 2,330	21,550 ± 3,180[b]
1,2-Diacyl GPE		
18:1–20:4	9,060 ± 1,990	7,670 ± 2,350[b]
16:0–20:4	12,400 ± 1,910	10,290 ± 2,560[b]
18:0–20:4	49,530 ± 2,510	42,100 ± 2,100[e]
1-O-alk-1′-enyl-2-acyl GPE		
18:1–20:4	1,010 ± 350	3,330 ± 1,520[c]
16:0–20:4	3,530 ± 780	11,510 ± 1,530[c]
18:0–20:4	4,740 ± 350	15,440 ± 2,110[c]

[a]Results are expressed as mean ± S.E.M. for 10^9 platelets from three different donors.

[b]Results are not significantly different from control values.

[c]$P < 0.001$.

[d]Results are given only for the whole subclass.

[e]$P < 0.05$.

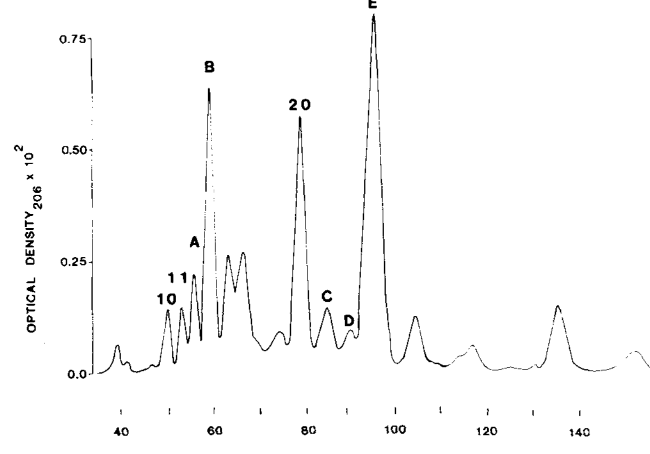

FIG. 2. Resolution of individual molecular species of platelet 1,2-diradyl GPE by HPLC. 1,2-Diradyl GPE obtained from 5×10^8 resting platelets was applied to the column. 1,2-Diacyl GPE peaks were labeled as in ref. 8. Identification of 1,2-diacyl molecular species: peak 10, 16:0–20:4; 11, 18:1–20:4; peaks between those labeled B and 20 are unidentified 1,2-diacyl molecular species; 20, 18:0–20:4. Identification of 1-O-alk-1′-enyl-2-acyl molecular species: peak A, 16:0 alk-1′-enyl-20:4; B, 18:1 alk-1′-enyl-20:4; peaks C and D not identified, E, 18:0 alk-1′-enyl-20:4.

species, alk-1′-enyl; 16:0–20:4, 18:1–20:4 and 18:0–20:4 GPE in resting platelets.

1,2-Diradyl GPC and GPE were also isolated from thrombin-stimulated cells. Following thrombin stimulation, the radioactivity in 1-radyl-2-acyl GPC decreased to 158,505 ± 16,513 (mean ± SEM, n = 6), or 56.5% of the starting value. The distribution in subclasses of 1-radyl-2-acyl GPC resolved as diglyceride acetates was 1,2-diacyl, 88.2%; 1-O-alkyl-2-acyl, 10.0%; and 1-O-alk-1′-enyl-2-acyl, 1.7%. For 1-radyl-2-acyl GPE, there was a significant increase of 15.1% in radioactivity from 81,522 ± 8,150 cpm to 93,750 ± 6,899 cpm ($p < 0.05$) after a 5-min treatment of the cells with thrombin. The distribution of radioactivity was 1,2-diacyl, 64.2%; 1-O-alkyl-2-acyl, 1.7%; and 1-O-alk-1′-enyl-2-acyl, 32.3%. In agreement with previous work, there was no change in 1,2-diradyl GPS while there was a substantial decrease in 1,2-diradyl GPI following thrombin stimulation, indicating that the response agonist included a net decrease in total [^{3}H]arachidonate content of platelet phospholipid (11,15,16).

The distribution of radioactivity in the arachidonoyl-containing molecular species of 1,2-diacyl GPC and GPE isolated from thrombin-stimulated cells and eluted by HPLC is shown in Figures 3 and 4 and Table 1. [^{3}H]-Arachidonate was decreased in all four molecular species of 1,2-diacyl GPC containing [^{3}H]arachidonate (Fig. 3 and Table 1). The 1-O-alkyl-2-arachidonoyl GPC-containing molecular species are also indicated in Figure 3 (as in Fig. 1) by small letters over arrows. The radioactivity associated with the 1-O-alkyl-2-[^{3}H]arachidonoyl molecular species of GPC (i.e., Fig. 3; b, c, d) was considerably less than that incorporated into 1-acyl-2-[^{3}H]-arachidonoyl GPC molecular species. Thrombin stimulation resulted in a very slight (not statistically significant) increase in the radioactivity of these molecular species of 1-O-alkyl-2-acyl GPC (Table 1). In thrombin-stimulated cells,

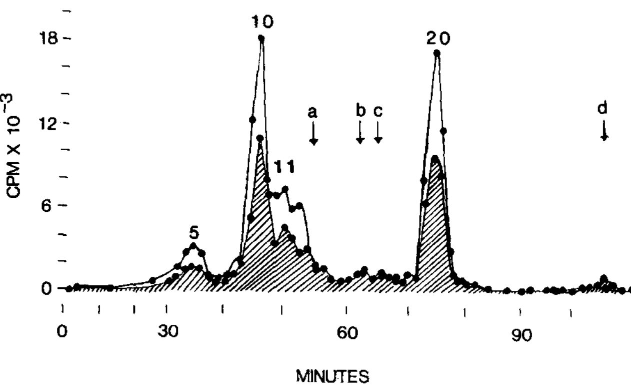

FIG. 3. Resolution of individual radiolabeled molecular species of 1,2-diradyl GPC from control or thrombin-stimulated (5 U/ml, 5 min, 37 C) platelets previously incubated for one hour at 37 C with [^{3}H]arachidonic acid. Shaded areas represent results from thrombin-stimulated platelets. Radioactivity was associated with peak 5, 18:2–20:4; 10, 16:0–20:4; 11, 18:1–20:4; and 20, 18:0–20:4. The level of incorporated [^{3}H]arachidonic acid in all these 1,2-diacyl arachidonoyl-containing molecular species is decreased by thrombin stimulation. Radioactivity in 1-O-alkyl-2-[^{3}H]arachidonoyl GPC molecular species (i.e., molecular species b, c and d of Figs. 1 and 3) is low and increases slightly following thrombin addition.

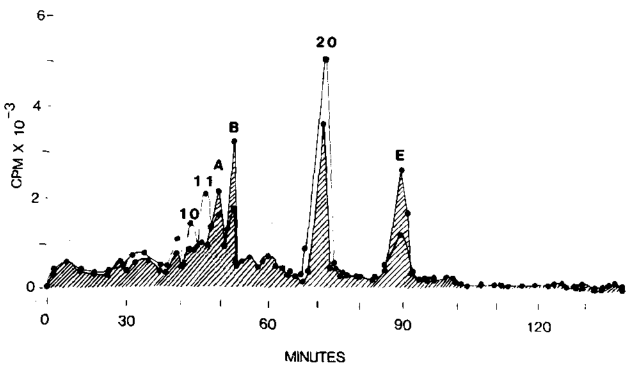

FIG. 4. Resolution of individual molecular species of 1,2-diradyl GPE from control and thrombin-stimulated (5 U/ml, 5 min 37 C) platelets previously incubated for 1 hr at 37 C with [^{3}H]arachidonic acid. Shaded areas represent thrombin results. Incorporated [^{3}H]arachidonoyl radioactivity was found in peaks 10, 11 and 20 (1,2-diacyl GPE); and A, B, and E (1-O-alk-1′-enyl-acyl GPE). There was a decrease in the radioactivity of peaks 10, 11 and 20 and a considerable increase in that of peaks A, B and E following thrombin stimulation.

the [^{3}H]arachidonate was decreased in 1,2-diacyl GPE arachidonoyl-containing molecular species (Fig. 4 and Table 1). The low level of radioactivity observed in 1-O-alk-1′-enyl GPE molecular species was increased over 300% following thrombin stimulation (Table 1 and Fig. 4).

In double labeling experiments using [^{14}C]arachidonic acid and [^{3}H]linoleic acid, the arachidonic acid was found to be incorporated into the same 1,2-diradyl GPC molecular species as seen previously (Figs. 1 and 3). [^{3}H]Linoleoyl radioactivity was found in peaks 5, 11, 12 and 21, which are the main linoleoyl-containing species of platelet 1,2-diacyl GPC, i.e., peak 5, 18:2–20:4; peak 11, 16:0–18:2; peak 12, 18:1–18:2; and peak 21, 18:0–18:2 GPC, respectively. Following thrombin addition, significant decreases were only found in the molecular species containing [^{3}H]arachidonate, as shown previously (data not shown). No significant incorporation of [^{3}H]linoleic acid into platelet 1,2-diradyl GPE was seen, in keeping with the low linoleic acid content of this phospholipid (1,2,5).

DISCUSSION

Liberation of arachidonic acid from its *sn*-2 linkage in membrane phospholipids is a necessary first step toward the production of thromboxane A_2 and lipoxygenase products in thrombin-stimulated human platelets (15,16). Previous workers have indicated that 1,2-diradyl GPI (11,17–19), 1,2-diradyl GPC (15,16,20) and 1,2-diradyl GPE (3,21) may all be considered potential sources of the arachidonic acid released for eicosanoid production.

Reverse phase HPLC was used in this study to resolve individual molecular species of phospholipid; it was effective in separating the various arachidonoyl-containing components. The incorporation of [^{3}H]arachidonic acid was highest in the 1,2-diacyl molecular species of both 1,2-diradyl GPC and 1,2-diradyl GPE. This agrees with previous results for human platelets (14) and, given the high radioactivity in 1,2-diacyl GPC and GPE molecular species, the identification of highly labeled molecular species was simplified. In general, the amount of incorporation was proportional to the mass of the molecular species within a given subclass, and these same phospholipids were those most rapidly deacylated after stimulation with thrombin. Ether phospholipids incorporated considerably less of the label in resting platelets, and the level of [^{3}H]arachidonate either increased slightly (1-O-alkyl-2-[^{3}H]arachidonoyl GPC; Fig. 3) or significantly (1-O-alk-1′-enyl-2-[^{3}H]arachidonoyl GPE; Fig. 4 and Table 1) following thrombin stimulation.

Previously we suggested that 1-O-alkyl-2-arachidonoyl GPC might be an important contributor of arachidonic acid in thrombin-stimulated cells (1). This had been shown for ionophore A23187-stimulated neutrophils (10). In view of the high specific activity of [^{3}H]arachidonate released by thrombin-stimulated platelets (22), the low acylation/deacylation of [^{3}Harachidonate in 1-O-alkyl-2-arachidonoyl GPC in resting or stimulated cells shown here and elsewhere (14) suggests that this ether lipid is not a significant source of arachidonic acid after addition of thrombin. Other workers have also demonstrated in other cells (23,24) and platelets (25,26) a more rapid uptake of arachidonic acid into 1,2-diacyl GPC and GPE compared to their ether subclasses. Ionophore-stimulated cells have shown a considerable decrease in 1-O-alkyl-2-[^{3}H]arachidonoyl GPC, and we have demonstrated the same for human platelets (14). However, there is no such decrease in thrombin-stimulated human platelets (14, and this work), although substantial deacylation of 1-O-alkyl-2-[^{3}H]arachidonoyl GPC does occur in thrombin-stimulated rabbit platelets (26).

The exact contribution of 1-O-alkyl-2-arachidonoyl GPC to the free arachidonate pool will be known only when mass changes in 1-O-alkyl-2-arachidonoyl GPC due to thrombin stimulation have been determined. 1-Acyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol is a minor contributor (15%) to the 30 nmol of arachidonic acid released from 10^9 thrombin-stimulated human platelets (11,27). In addition, only picomole levels of

platelet activating factor are generated by the same number of stimulated cells (28,29). Thus, we conclude that the pathway for the production of platelet activating factor, which involves deacylation of 1-O-alkyl-2-acyl GPC followed by acetylation, is not a significant source of arachidonic acid in human platelets (14). On the other hand, 1,2-diacyl molecular species of GPC and GPE appear crucial with respect to the release of arachidonic acid. Deacylation of the various molecular species of 1,2-diradyl GPC and GPE appears related to the rate of uptake in the resting cell and occurs most markedly in 1,2-diacyl phospholipids. Thus, 1-palmitoyl-2-arachidonoyl GPC, 1-steroyl-2-arachidonoyl GPC and 1-stearoyl-2-arachidonoyl GPE are the main molecular species involved in acylation with [^{3}H]arachidonic acid in resting cells, as well as deacylation in stimulated cells. The lack of deacylation in those molecular species of 1,2-diacyl GPC radiolabeled with linoleic acid but not [^{14}C]arachidonic acid argues for selectivity in the deacylation of arachidonoyl vs non-arachidonoyl-containing molecular species of 1,2-diacyl GPC.

In this study we found that the slight increase in total GPE radioactivity after 5 min of thrombin stimulation was due to an increase in 16:0, 18:1 and 18:0 alk-1′-enyl-2-[^{3}H]arachidonoyl GPE. Thus, the small increase in 1,2-diradyl GPE radioactivity represented a small decrease of radioactivity in 1,2-diacyl GPE (particularly 1-stearoyl-2-[^{3}H]arachidonoyl PE) and a greater increase in plasmalogen structures. Transacylation between 1,2-diacyl GPC and plasmalogen GPE of human platelets has been reported previously (30,31). The considerable increase in the radioactivity of 1-O-alk-1′-enyl-2-acyl GPE indicates that this ether phospholipid may also be a significant source of arachidonate.

It was not possible in this study to determine exact mass changes in individual molecular species of platelet phospholipid following addition of thrombin. A decrease in the optical density at 206 nm for 18:2-20:4 (peak 5), 16:0-20:4 (peak 10), 18:1-20:4 (peak 11) and 18:0-20:4 (peak 20) GPC was observed, while changes in other molecular species were not apparent. However, it is difficult to determine mass changes when one monitors unsaturation at 206 nm.

In summary, our work indicates arachidonoyl-containing molecular species of 1,2-diacyl GPC and GPE are the main source of [^{3}H]arachidonic acid liberated in thrombin-stimulated platelets, particularly compared to 1-O-alkyl-2-arachidonoyl GPC and probably 1-O-alk-1′-enyl-2-arachidonoyl GPE. The main action of endogenous acyl hydrolase is on the two main 1-acyl-2-arachidonoyl molecular species of GPC as well as on 1-stearoyl-2-arachidonoyl GPE. Transacylation involves mainly three molecular species, 16:0, 18:1, and 18:0 alk-1′-enyl-2-[^{3}H]arachidonoyl PE.

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Oleic Acid Modulates the Partitioning of Cholesterol from Micellar Bile Salt Solution

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The effects of monounsaturated fatty acid (oleic acid) on cholesterol monomer activity and on the rate of cholesterol influx were studied in vitro. A polyethylene disc method was employed to determine cholesterol monomer activity in constant sodium taurocholate-cholesterol micellar solution containing different oleic acid concentration levels at pH 5.5, 6.5 and 7.2. In addition, the effect of oleic acid on the rates of cholesterol influx was determined using an everted rat jejunal sac technique.

At pH 5.5, increased oleic acid concentration from 5 to 10 mM resulted in significant decreased apparent cholesterol monomer activity (3.8 ± 0.21 nmol/disc vs 1.0 ± 0.08, $P < 0.001$). At pH 6.5, apparent cholesterol monomer activity was 2.3 ± 0.19 nmol/disc at 5 mM and 0.5 ± 0.09 at 16 mM oleic acid level ($P < 0.001$). Apparent monomer activity of cholesterol in micellar solutions at pH 7.2 used for the influx study at 5 and 15 mM oleic acid concentration level was 1.8 ± 0.14 and 0.7 ± 0.08 nmol/disc, respectively ($P < 0.001$). Thus there was a significant decrease in cholesterol monomer activity by the addition of oleic acid at each pH. The rate of cholesterol influx across the brush border membrane of the rat jejunum at 5 and 15 mM oleic acid concentration level was 3.2 ± 0.31 and 1.5 ± 0.21 nmol/100 mg dry weight tissues/min, respectively ($P < 0.001$).

It is concluded that the addition of oleic acid decreases both monomer activity and the rate of influx of cholesterol from micellar solution. This effect is primarily attributable to the inhibition of the release of cholesterol monomers from the mixed micelle.
Lipids 22, 121–124 (1987).

Despite the fact that the partitioning of cholesterol from micellar bile salt solution is modulated by the micellar constituents (1–5) and pH (6), little study has been done on the role of fatty acids in the physiology of cholesterol in micellar solution. Hollander and Morgan (3) showed that both oleic acid and lecithin inhibited cholesterol absorption in the rat in vivo. On the other hand, Thomson and Cleland (4) demonstrated that palmitic acid increased and inhibited cholesterol uptake in vitro when lecithin was absent and present, respectively. Thus, the effect of fatty acids on the physiology of cholesterol in micellar bile salt solution is still contradictory.

Since free fatty acids are present in bile (7) and in the intestinal bulk phase following lipolysis (8), it is important to evaluate the effect of fatty acids on the partitioning of cholesterol out of the micelle.

Accordingly, the aim of this study was to examine the effect of oleic acid, one of the predominant monounsaturated fatty acids, on the partitioning of cholesterol from micellar solutions at various physiological pH (pH 5.5, 6.5 and 7.2). In addition, the effect of oleic acid on the rate of influx of cholesterol across the brush border membrane was studied using the everted rat jejunal sac technique in vitro.

MATERIALS AND METHODS

Experimental design. Experiments were designed for two purposes. First, the effect of oleic acid on cholesterol monomer activity in 30 mM sodium taurocholate-0.1 mM cholesterol micellar solution was studied at pH 5.5 and 6.5. Polyethylene disc technique was used to determine the partitioning of cholesterol from micellar solution into the disc (apparent monomer activity). Second, the rates of influx of cholesterol from a similar micellar solution with 5 or 15 mM oleic acid concentration level, pH 7.2, were investigated to confirm the altering effect of oleic acid on the influx through the brush border membrane and compared the relationship between apparent monomer activity and the rate of influx of cholesterol. The rate of cholesterol influx was determined using an everted rat jejunal sac method in vitro.

Chemicals. Sodium taurocholate (NaTC) was purchased from ICN (K and K Laboratories, Plainview, New York). Oleic acid was obtained from Nu-Chek-Prep (Elysian, Minnesota) and cholesterol from Fisher Scientific (Springfield, New Jersey). [1,2-^{3}H]Cholesterol, 53.0 Ci/mol, was purchased from New England Nuclear (Boston, Massachusetts). All were >98% pure as judged by thin layer chromatography and were used without further purification. ^{14}C-Polyethylene glycol (PEG; MW 4,000; sp act 34.7 mCi/mmol) was obtained from Amersham (Arlington Heights, Illinois) and was used as supplied by the manufacturer. All other reagents were of analytical grade.

Micellar solutions. Three kinds of micellar sodium taurocholate solutions were prepared: a) 30 mM NaTC, 0.1 mM ^{3}H-cholesterol (sp act 1 μCi/μmol) and 5 or 10 mM oleic acid, final pH 5.5; b) 30 mM NaTC, 0.1 mM ^{3}H-cholesterol and 5 or 16 mM oleic acid in lactated Ringer having the composition 130 mEq/l Na^+, 4 mEq/l K^+, 3 mEq/l Ca^{++}, 109 mEq/l Cl^-, 28 mEq/l lactate$^-$, final pH 6.5. Ten and 16 mM oleic acid concentrations used were close to the maximal micellar solubility at each pH level (6); c) 30 mM NaTC, 0.1 mM ^{3}H-cholesterol and 5 or 15 mM oleic acid were prepared in 0.02 M phosphate buffer, pH 7.2, with 0.12 M NaCl. Since the fatty acid is present in the upper small intestine under these physiological conditions, the current experiments do not provide a fatty acid-free solution.

Apparent cholesterol monomer activity. The partitioning of cholesterol from the various micellar solutions described above was determined as previously reported (6) based on the method of Sallee (9). Briefly, polyethylene discs, 0.6 cm in diameter punched out from polyethylene film 0.006 inches thick, were a gift from Verney L. Sallee (Alcon Laboratories, Fort Worth, Texas). The discs were washed with methanol and distilled water and were dried before use. Partitioning of cholesterol into polyethylene discs from micellar solutions was determined after equilibration for 24 hr. Each disc was placed in 3 ml micellar solution in a 25 mm × 100 mm screw-capped tube and incubated at 37 C in a Dubnoff incubator at 100

oscillations/min. After an equilibration period, the disc was removed and rinsed thoroughly in the distilled water. The polyethylene disc was placed in the scintillation vial and 0.5 ml of distilled water was added; 0.5 ml of the original micellar solution was used to determine the specific activity of cholesterol. Discs and micellar solutions were counted simultaneously following the addition of 13 ml scintillation solution and partitioning of cholesterol into the disc was expressed as nmol/disc.

Effect of oleic acid on the rate of cholesterol influx. Male Wistar rats, 180–200 g, were fasted overnight and killed by decapitation. A section of proximal jejunum (ca. 25 cm from Lig. Treitz) was removed immediately and rinsed with ice-cold physiological saline. The intestine was everted immediately over a glass rod and gently filled with cold 0.02 M phosphate buffer, pH 7.2, with 0.12 M NaCl (0.5 ml/2.5 cm gut). Sacs, 2.5 cm long, were prepared by cutting double ligatures and were rinsed immediately with cold 0.02 M phosphate buffer with 0.12 M NaCl. The sacs were randomized and each was immersed in oxygenated micellar solution in an Erlenmyer flask. All flasks were gassed with 95% O_2 5% CO_2 and incubated at 37 C in a Dubnoff metabolic shaking incubator at 100 oscillations/min for 4 min. The incubation medium consisted of 10 ml of a bile salt micellar solution that contained 300 μmol of sodium taurocholate, 50 or 150 μmol of oleic acid and 1 μmol of ^{3}H-cholesterol with a tracer amount of ^{14}C labeled PEG (0.5 μCi/10 ml). At the end of the incubation period, sacs were removed and rinsed vigorously for 20 sec in iced 0.02 M phosphate buffer, pH 7.2, with 0.12 M NaCl and placed on moistened filter paper. The ends of the sacs were transected and the central cylinder of the intestine was dried overnight in an oven at 60 C. Part of the sac was preserved in buffered 10% formalin for histological examination. Dried sacs were weighed, placed in glass scintillation vials and dissolved in 0.5 ml of 1 N NaOH in an oven at 120 C for 30 min. After neutralization with 0.5 ml 1 N HCl, 13 ml scintillation solution (phase-combining system, New England Nuclear) was added to determine the radioactivity.

Preliminary validation experiments demonstrated that the water-soluble nonabsorbable marker (PEG) was homogeneously distributed in the adherent mucosal fluid layer after a 4-min incubation period and constant thereafter. Cholesterol influx (nmol/100 mg dry wt) was a linear function of the incubation time for at least the 4-min incubation period, and the intercept of the linear regression curve was nearly zero at 0 time for both high and low concentration of oleic acid. The cholesterol taken up by the tissue was not esterified during a 4-min incubation period. Accordingly, a 4-min incubation period was selected in the present study. The rate of cholesterol influx was calculated without correction for adherent mucosal fluid volume (10). Since the concentration of cholesterol in the adherent mucosal fluid layer was extremely low as the result of continuous uptake by the tissue, the correction was not necessary under the present experimental condition. The values were expressed as nmol/100 mg dry wt/min.

Effect of oleic acid on cholesterol solubility. In the fundamental study, 30 mM sodium taurocholate, an excess amount of cholesterol (3 mM) and 0, 5, 10 and 15 mM oleic acid were mixed in 0.02 M phosphate buffer, pH 7.2, with 0.12 M NaCl solution and incubated at 37 C for more than three days. These solutions were filtered through a sterile 0.2-μm filter (Nihon Millipore, Tokyo, Japan) and 0.5 ml of the filtrate was placed in a 10-ml volumetric flask. Coprostanol was added as an internal standard and filled up to 10 ml with methanol; 0.5 ml of this solution was evaporated, and trimethylsilyl (TMS) ether derivatization was made and subjected to gas liquid chromatography (Shimadzu Seisakusho, Kyoto, Japan) equipped with a Van den Berg's solventless injector, WCOT column, 25 m × 0.35 mm id, coated with SE-30 (LKB-Produktor, Stockholm, Sweden) and flame ionization detector. The temperature of the column and injection port was 280 C and 310 C, respectively, and the flow rate of helium carrier gas was 2 ml/min. Cholesterol was quantified by calculating a peak area ratio to internal standard.

Determination of radioactivity. Radioactivity was counted in a Beckman M52 scintillation counter and determined by means of an external standardization technique to correct for variable quenching of the two isotopes.

Statistics. Values were mean ± SEM for at least six observations. Student's t-test was used, and p values less than 0.05 were considered to be significant.

RESULTS

Apparent cholesterol monomer activity. Apparent cholesterol monomer activity in micellar solutions at pH 5.5 and 6.5 is depicted in Figure 1. Polyethylene discs were equilibrated after a 24-hr incubation period. The higher the cholesterol monomer activity after the equilibration period, the higher the monomer activity throughout the incubation period. Apparent cholesterol monomer activity in micellar solutions after equilibration is shown in Figure 2. Apparent cholesterol monomer activity in the constant micellar solution (30 mM NaTC, 5 mM oleic acid and 0.1 mM cholesterol) was significantly higher at pH

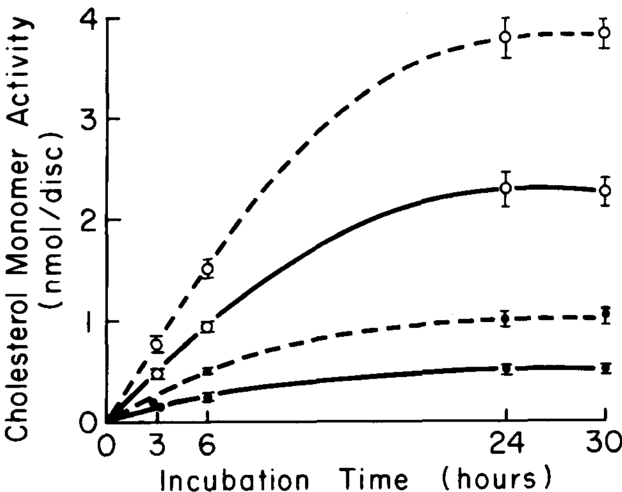

FIG. 1. Effect of oleic acid and pH on apparent cholesterol monomer activity in micellar solutions during various incubation periods. ○, Cholesterol monomer activity in 30 mM NaTC, 5 mM oleic acid and 0.1 mM cholesterol mixed micellar solution. ●, Monomer activities of cholesterol in similar micellar solutions except for oleic acid concentrations (10 mM at pH 5.5 and 16 mM at pH 6.5). – – –, pH 5.5; ——, pH 6.5. Values are mean ± SEM of 6 observations.

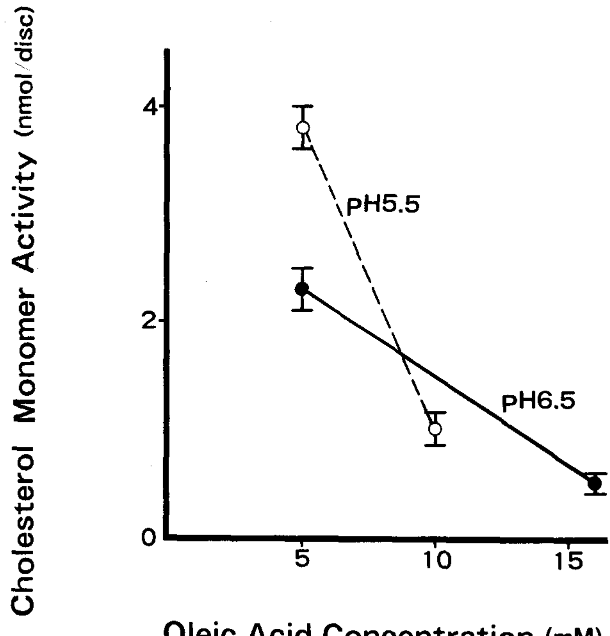

FIG. 2. Effect of oleic acid on apparent cholesterol monomer activity in micellar solution. Micellar solutions contained 30 mM NaTC, 0.1 mM cholesterol and oleic acid below its maximal micellar solubility at pH 5.5 and pH 6.5. ○, Apparent cholesterol monomer activity in micellar solutions with 5 mM and 10 mM oleic acid at pH 5.5 (– – –); ●, apparent cholesterol monomer activity at 5 and 16 mM oleic acid at pH 6.5 (——). Values represent mean ± SEM (n = 6).

5.5 than at pH 6.5 (3.8 ± 0.21 vs 2.3 ± 0.19 nmol/disc, $p < 0.001$), which confirmed our previous observations (6). Next, only oleic acid concentration was increased close to its saturation point at each pH level. At each pH, the higher the oleic acid concentration, the lower the monomer activity of cholesterol. At pH 5.5, increased oleic acid concentration from 5 to 10 mM decreased apparent cholesterol monomer activity from 3.8 ± 0.21 to 1.0 ± 0.08 nmol/disc ($p < 0.001$). Similarly, at pH 6.5, elevating oleic acid concentration decreased apparent cholesterol monomer activity significantly (2.3 ± 0.19 nmol/disc at 5 mM oleic acid and 0.5 ± 0.09 at 16 mM oleic acid concentration, $p < 0.001$). These results indicate that cholesterol monomer activity declines with increasing oleic acid concentration at constant cholesterol concentration level (Fig. 2).

In the second experimental series, apparent cholesterol monomer activity was determined in micellar solutions (30 mM NaTC, 0.1 mM ^{3}H-cholesterol with 5 or 15 mM oleic acid, pH 7.2) used for the following influx study to confirm the effect of oleic acid on apparent cholesterol monomer activity and on the rate of cholesterol influx. Apparent cholesterol monomer activity was 1.8 ± 0.14 nmol/disc at 5 mM oleic acid level and 0.7 ± 0.08 at 15 mM oleic acid level, respectively ($p < 0.001$). Again, the apparent monomer activity of cholesterol was significantly lower at the higher oleic acid concentration level.

Rate of cholesterol influx. The rates of cholesterol influx across the brush border membrane of rat jejunal sac exposed to the 30 mM NaTC-0.1 mM cholesterol micellar solution with 15 mM and 5 mM oleic acid were 1.5 ± 0.21 and 3.2 ± 0.31 nmol/100 mg dry weight tissue/min, respectively (Fig. 3). There was a significant difference ($p < 0.001$) and the rate of influx was higher at 5 mM oleic acid concentration level than at 15 mM oleic acid concentration. Histological examination following the exposure to the micellar solution showed no damage to the mucosa.

Effect of oleic acid on cholesterol solubility. Increased oleic acid concentration resulted in higher cholesterol solubility (Fig. 4); 0.2 ± 0.03 mM (SEM) cholesterol was solubilized in 30 mM NaTC solution without oleic acid. The cholesterol solubility was 0.5 ± 0.06 mM, 1.4 ± 0.19 mM and 2.6 ± 0.22 mM in micellar solutions containing 5, 10 and 15 mM oleic acid, respectively.

DISCUSSION

Cholesterol is passively taken up by the enterocyte as monomers in the intermicellar aqueous phase in equilibrium with micellar aggregate (11,12). Cholesterol monomer in the intermicellar aqueous phase is governed by its partition coefficient between aqueous phase and micellar aggregate.

This partition coefficient mainly depends on the constituents of the micelle. Phospholipid inhibits the uptake and the partitioning of cholesterol from micellar solution (1-3). The effect of fatty acid on the uptake of cholesterol by the enterocyte has been studied, and contrary results were reported. Thomson and Cleland (4) reported that palmitic acid enhances the uptake of cholesterol. On the other hand, Hollander and Morgan (3) demonstrated that oleic acid decreases cholesterol absorption.

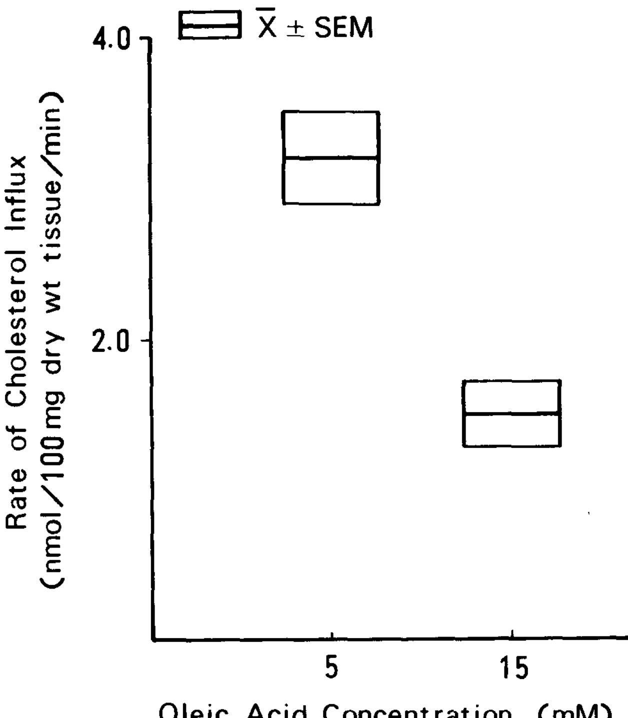

FIG. 3. Effect of oleic acid on the rate of cholesterol influx across the brush border membrane of everted rat jejunum in vitro. Everted sacs were exposed to micellar solution containing 30 mM NaTC, 0.1 mM cholesterol with 5 or 15 mM oleic acid at pH 7.2 and were incubated for 4 min.

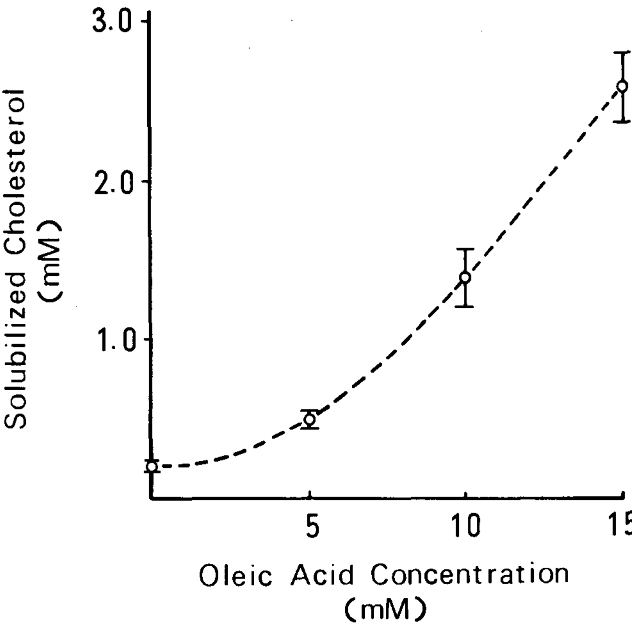

FIG. 4. Effect of oleic acid on maximal micellar solubility of cholesterol. Excess amounts of cholesterol were dissolved in 30 mM NaTC with 0, 5, 10 and 15 mM oleic acid at pH 7.2. Solutions were mixed well and incubated at 37 C for more than 3 days to reach equilibrium. Clear filtrate through the membrane (0.2 μm) was subjected to gas liquid chromatography. Values are mean ± SEM.

In the present study, two factors (micellar solubility and the size of the micelle) are possibly modulated by the presence of oleic acid. First, the addition of oleic acid enhanced the micellar solubility of cholesterol, as shown in the present experiment. In the additional study, the addition of small amount of oleic acid below 5 mM concentration level had no significant effect on the partitioning of cholesterol from micellar solutions. Increased micellar solubility by the addition of phospholipid (1) and by elevating the pH (6) resulted in lower monomer activities of fatty acids and cholesterol. The present study revealed the evidence that increased micellar solubility of cholesterol by the addition of oleic acid results in decreased apparent cholesterol monomer activity (partitioning of cholesterol) (Figs. 1 and 2). Apparent cholesterol monomer activity in the micellar solution at a constant cholesterol concentration level mainly depends on its partition coefficient between aqueous phase and micellar aggregate, which regulates cholesterol monomer concentration in the intermicellar aqueous phase. The lower monomer activity at the higher oleic acid concentration presumably can be ascribed to the lower partition coefficient by forming the mixed micelle, which can incorporate much more cholesterol inside the micelle. Thus, the addition of oleic acid in the sodium taurocholate-cholesterol micellar solution shifts cholesterol monomers away from aqueous phase to micellar aggregates.

Another possible factor is the size of the micelle. The addition of oleic acid increases the size of the micelle (13). Expanded micelles diffuse toward the brush border membrane at a slower rate, which declines the rate of cholesterol influx.

The current study was focused on the role of monounsaturated fatty acid (oleic acid) in the partitioning phenomenon of cholesterol in the micellar solutions. Increased oleic acid concentration levels resulted in the decreased apparent cholesterol monomer activity, which positively correlated with the rate of cholesterol influx across the brush border membrane in vitro. The overall effect of oleic acid on cholesterol absorption in vivo should be examined further, since fatty acids are efficiently absorbed (12) and affect the esterification and enhance the transport of cholesterol (5,14,15). The results support the observations by Hollander and Morgan (3), but are opposite to the report by Thomson and Cleland (4). This might be due to the difference in fatty acids, because Thomson and Cleland used palmitic acid. Study of the effect of fatty acid type on the partitioning of cholesterol out of micelle is in progress. Of particular interest is the modulating effect of free fatty acids on the partitioning of cholesterol from micellar solutions into the organic phase (polyethylene disc). Since free fatty acids are present in bile (7) and gallstone (16), the role of free fatty acids in the pathogenesis of gallstone might be deducible.

It is concluded from this study that the lowering effect of monounsaturated fatty acid (oleic acid) on the partitioning and rate of influx of cholesterol from micellar bile salt solution in vitro is mainly mediated by its enhanced micellar holding capacity for cholesterol, which would shift cholesterol monomers from aqueous phase to mixed micelles.

ACKNOWLEDGMENTS

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METHODS

Electrochemical Detection of Phospholipid Hydroperoxides in Reverse-Phase High Performance Liquid Chromatography

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Hydroperoxy derivatives of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) can be separated from their respective phospholipids by reverse-phase high performance liquid chromatography (HPLC). However, ultraviolet absorption due to conjugated diene cannot detect the hydroperoxy group. In this work, an electrochemical (EC) detector was first applied to the analysis of hydroperoxy phospholipids. Both the PC and PE hydroperoxides from rat liver were reduced quantitatively by the glassy carbon electrode at −300 mV vs Ag/AgCl. Since neither the hydroxy derivatives nor unoxidized phospholipids showed any response, it would seem this technique can be used to distinguish phospholipid hydroperoxides from their hydroxy derivatives. Thus, the reverse phase HPLC-EC detection method is proposed for the specific analysis of hydroperoxy phospholipids in biological tissues.
Lipids 22, 125–128 (1987).

In recent years, lipid peroxidation has attracted much interest in relation to the aging process, cancer development and other pathological conditions. To measure lipid hydroperoxides, the primary products of lipid peroxidation in the biological system, sensitive methods have been developed which use fluorescence (1), chemiluminescence (2,3), peroxidase (4,5) and cyclooxygenase activity (6).

Membrane phospholipids including phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are assumed to be the main species responsible for hydroperoxide formation during lipid peroxidation in vivo. PC hydroperoxides can be separated from unoxidized PC by reverse-phase high performance liquid chromatography (HPLC) (7–10). Thus, reverse-phase HPLC seems to be useful as a direct method for determining lipid hydroperoxides. Previously, we succeeded in separating the PC and PE hydroperoxides of rat liver from their respective phospholipids by reverse-phase HPLC and applied the technique to measure the phospholipid peroxidation products of rat liver after carbon tetrachloride administration (11). However, we found it difficult to distinguish phospholipid hydroperoxides from their hydroxy derivatives on the chromatogram because the detection was based on UV absorption due to conjugated diene structure. A method that would specifically detect the hydroperoxy group was required in order to use the HPLC technique for the measurement of lipid hydroperoxides. Moreover, it may be essential to distinguish phospholipid hydroperoxides from the hydroxy derivatives because enzymatic reduction of PC hydroperoxides is implied in the cellular defense system against lipid peroxidation (12).

We previously conducted a polarographic study of triacylglycerol hydroperoxides and obtained polarographic reducing waves under a dropping mercury electrode (13,14). An electrochemical (EC) detector equipped with a glassy carbon electrode has recently been developed, a significant improvement since Kissinger et al. (15) first proposed combining HPLC with the EC detector. Therefore, we investigated the effectiveness of the HPLC-EC detector in detecting phospholipid hydroperoxides.

Results showed that phospholipid hydroperoxides could be detected quantitatively by the EC detector, while there was no response from the hydroxy derivatives and unoxidized phospholipids. Compared with the UV detector for phospholipid hydroperoxides, the EC detector seems to have a higher sensitivity.

EXPERIMENTAL

Materials. Male Wistar rats, 8 wk old, were used. Rats were anesthetized, and whole livers were removed and washed with normal saline and then kept at −80 C prior to lipid extraction. PC and PE were obtained from the liver lipid fraction according to the method described previously (16). Hydroperoxy and hydroxy derivatives of rat liver PC and PE were prepared from their respective photosensitized oxidation products by the procedure described previously (16). Hydroperoxy derivatives of egg yolk PC were obtained by the same method used for rat liver PC.

The amount of the sample used in the experiments was obtained by successive dilution of the original weighed sample.

Apparatus. A Shimadzu liquid chromatograph LC-4A with a YMC A-212-C-8 column (6 × 150 mm, 5 μm particle size; Yamamura Kagaku Co., Kyoto, Japan) was used for HPLC analysis. A Shimadzu SPD-2A variable-wavelength UV detector and an Irica E-502 amperometric detector (Irica Co., Kyoto, Japan), which contains a glassy carbon electrode, were used as the UV and EC detectors, respectively. The EC detector was connected in series behind the UV detector. The chromatograms were recorded by a Shimadzu Chromatopack C-R1B or C-R3A processor. The glassy carbon electrode was washed and polished after a run of ca. 40 hr. Before the analysis, a 2-hr run was necessary to stabilize the electrode.

Reverse-phase HPLC. The sample was dissolved in a mixture of methanol and chloroform (93:7, v/v), and 150 μl was injected into the HPLC column. The column was eluted with methanol/water/acetonitrile (90.5:7:2.5, v/v/v) containing 0.25 mM sodium perchlorate. The flow rate was kept at 1.8 ml/min. The eluent was monitored at 235 nm for UV detection and −300 mV vs Ag/AgCl for EC detection.

The mobile phase was kept free from oxygen by continuous bubbling through helium gas. No attempt was made to remove oxygen from the sample solution.

RESULTS

Figure 1 shows typical chromatograms of egg yolk PC hydroperoxides obtained by the UV and EC detectors.

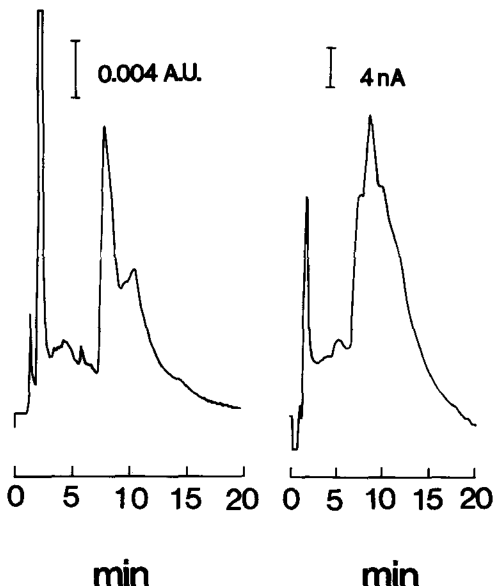

FIG. 1. Chromatograms of phosphatidylcholine hydroperoxides obtained by UV (A) and EC detection (B). The amount of PC hydroperoxides applied on the column was 50 nmol. The elution solvent contained 0.25 mM sodium perchlorate. Applied electrode potential was −400 mV vs Ag/AgCl. Wavelength of UV detector was adjusted at 235 nm.

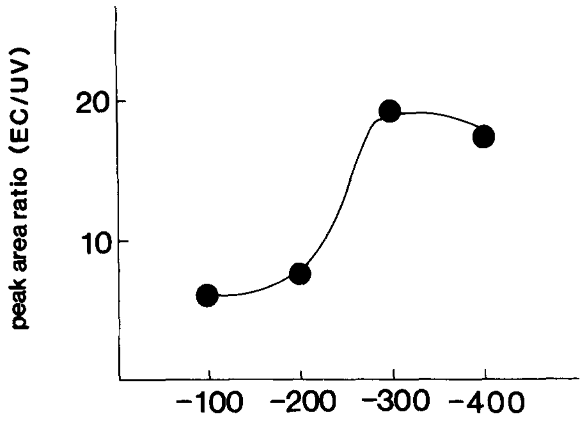

FIG. 2. Electric current-electrode potential curve. Experimental conditions were the same as those for Fig. 1, except the amount of phosphatidylcholine hydroperoxides applied on the column was 70 nmol and applied electrode potentials were changed. The electric current was represented as the ratio of the peak area obtained by EC detection against that obtained by UV detection at 235 nm.

In both chromatograms, the hydroperoxides appeared as overlapping peaks at a retention time of ∿7–10 min.

The ratio of the peak area obtained by EC detector to that obtained by UV detector was calculated at differing electrode potentials ranging from −100 to −400 mV vs Ag/AgCl, as shown in Figure 2. The EC detector was found to be the most sensitive at a potential of −300 mV vs Ag/AgCl in the above range. Figure 3 shows the effect of sodium perchlorate concentration on the sensitivity of

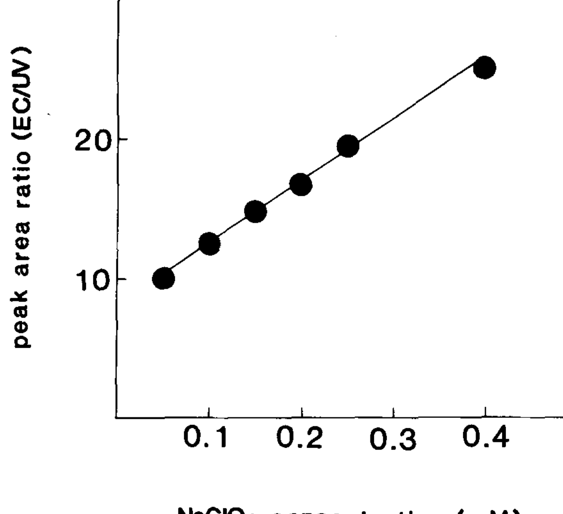

FIG. 3. Effect of concentration of supporting electrolyte on the sensitivity of EC detector. The experimental conditions were the same as those for Fig. 1, except the amount of phosphatidylcholine hydroperoxides applied was 70 nmol and the salt concentrations of the elution solvent were changed.

the EC detector. Sensitivity increased linearly with the increase in salt concentration. However, the higher concentration was found to generate large noises because the salt adhered to the surface of the electrode, and sudden lowering of the sensitivity in shorter periods resulted.

From the results of Figures 2 and 3, −300 mV vs Ag/AgCl and 0.25 mM were selected as the electrode potential and the supporting electrolyte concentration for the following experiments.

Under these analytical conditions, PC hydroperoxides of rat liver gave prominent peaks on the chromatogram monitored by the EC detector, similar to that of the UV detector (Fig. 4). Hydroxy derivatives of rat liver PC were eluted at almost the same position as the hydroperoxy derivatives. However, there were no peaks for the hydroxy derivatives on the chromatogram monitored by the EC detector. Unoxidized PC was eluted later than the hydroperoxy and hydroxy derivatives, as indicated on the chromatogram by the UV detector based on nonspecific end absorption (203 nm). Unoxidized PC was not detected by EC detection.

Similar results were obtained from rat liver PE and its hydroperoxy and hydroxy derivatives, but only the hydroperoxy derivatives gave prominent peaks on the chromatogram obtained by the EC detector (Fig. 5).

For both rat liver PC and PE, a linear relationship was observed between the amounts of their hydroperoxides and the peak areas in the chromatograms obtained by EC and UV detections in the range from 1 to 25 nmol (Figs. 6 and 7) (correlation coefficient: 0.990 for PC hydroperoxides by EC detection, 0.999 for PC hydroperoxides by UV detection, 0.823 for PE hydroperoxides by EC detection, 0.980 for PE hydroperoxides by UV detection). The slope of the regression lines indicates that the sensitivity of EC detection is more than 10 times higher than that of the UV detection for both phospholipid hydroperoxides. Conjugated diene isomers occupied more than 70% of the

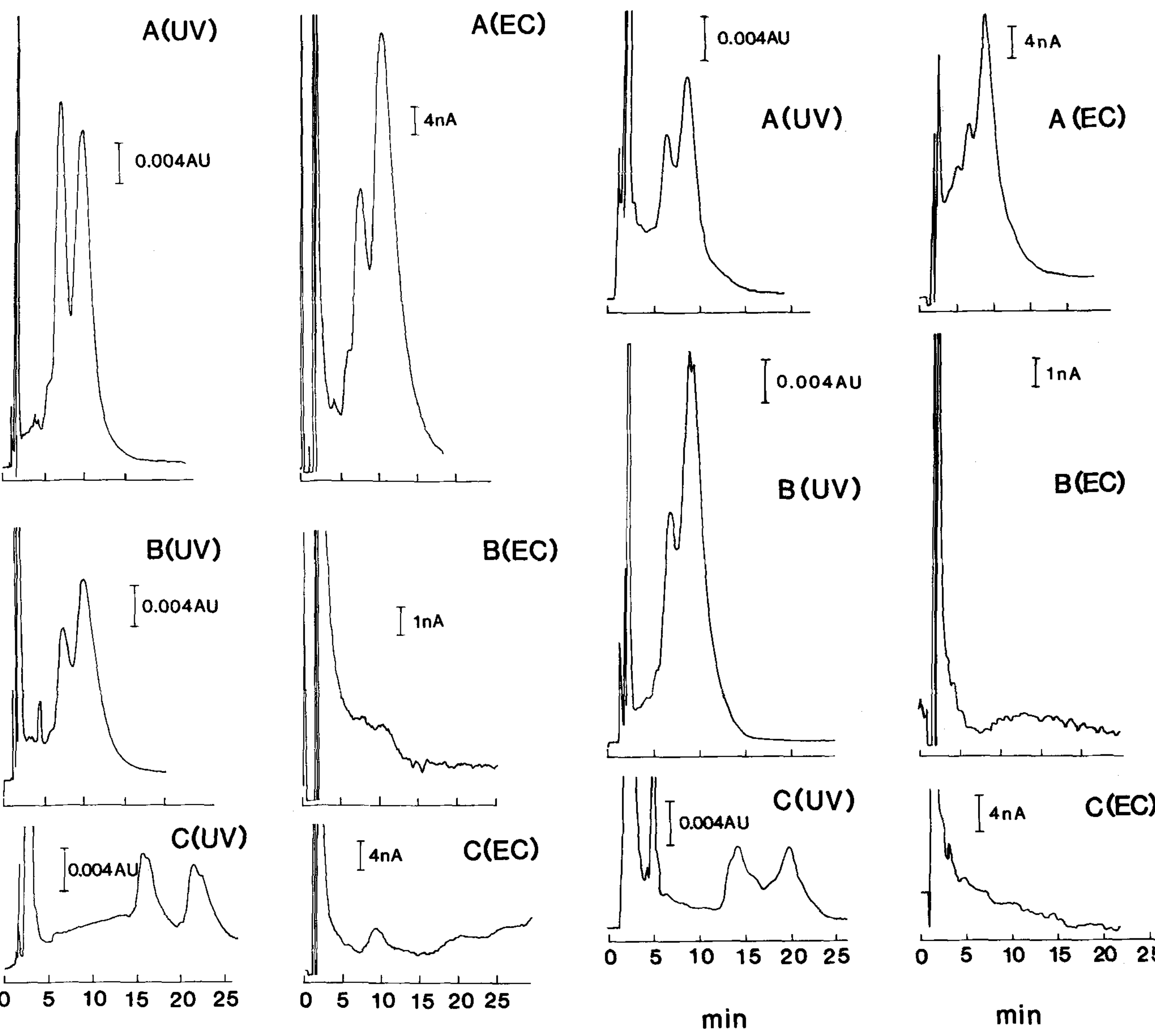

FIG. 4. Chromatograms of hydroperoxy and hydroxy derivatives of rat liver phosphatidylcholine (PC) and unoxidized PC. (A) Hydroperoxy derivatives (40 nmol), (B) hydroxy derivatives (20 nmol) and (C) unoxidized PC (20 nmol) were injected into the column. The eluting solvent contained 0.25 mM sodium perchlorate. Applied electrode potential was −300 mV vs Ag/AgCl. The UV detector was adjusted at 235 nm for hydroxy and hydroxy derivatives, and 203 nm for unoxidized PC.

FIG. 5. Chromatograms of hydroperoxy and hydroxy derivatives of rat liver phosphatidylethanolamine (PE) and unoxidized PE. (A) Hydroperoxy derivatives (20 nmol), (B) hydroxy derivatives (60 nmol) and (C) unoxidized PE (20 nmol) were injected into the column. Experimental conditions were the same as those for Fig. 4.

preparations used for standard phospholipid hydroperoxides (16), although they involved nonconjugated diene isomers, which might be undetectable by the absorption at 235 nm. Therefore, the scales of the peak area obtained by the UV absorption in Figures 6 and 7 were corrected by assuming that the conjugated diene isomers exclusively existed in the sample. The lowest detectable level of the EC detector was determined to be 200 pmol for PC hydroperoxides and 200 pmol for PE hydroperoxides.

DISCUSSION

Many attempts have been made to detect biological materials using the EC detector with HPLC, a combination that was proposed by Kissinger et al. (15). However, there are only a few studies on the use of the EC detector for the oxidized form of hydrophobic compounds. We previously found the reduction of lipid hydroperoxides by polarography (13,14) and thus were motivated to investigate the effectiveness of the EC detector for HPLC analysis of lipid hydroperoxides. The analytical capability of the EC detector seems to be limited when this detector is applied to hydroperoxy phospholipids. A higher concentration of salt, which would strengthen the supporting electrolyte, might elevate the response of the detector. However, we used a lower concentration of salt for this analysis because the electrode could not be stabilized for a long time. On the other hand, a higher electrode potential (−300 mV vs Ag/AgCl) was chosen because the lower electrode potential overlapped with that of oxygen.

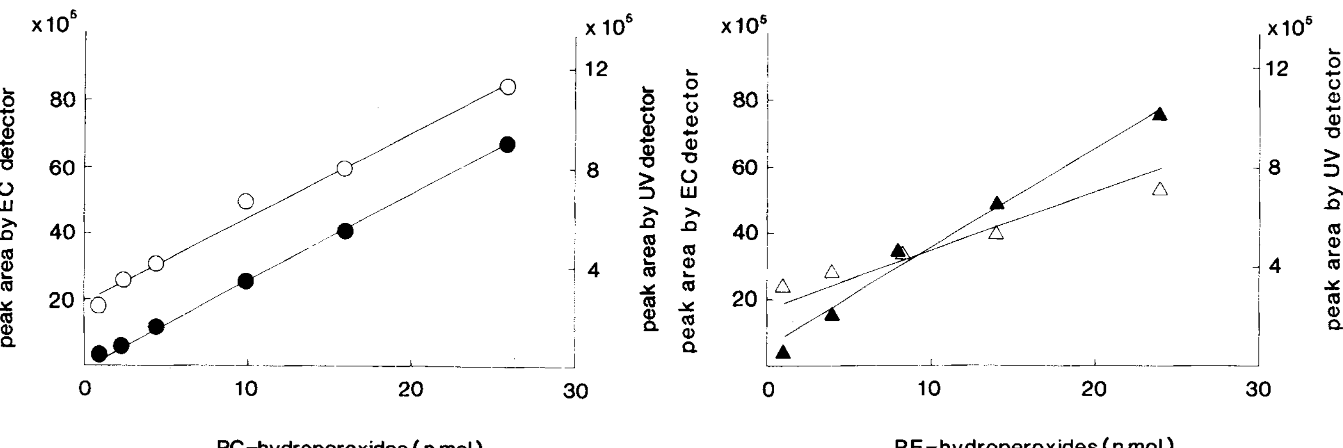

FIG. 6. Calibration curves for PC hydroperoxides. Experimental conditions were the same as those for Fig. 4. The scale of UV absorption was adjusted as the amount of conjugated diene isomers in the sample was estimated to be 74% (16). ●, UV detection; ○, EC detection.

FIG. 7. Calibration curves for phosphatidylethanolamine hydroperoxides. Experimental conditions were the same as those shown in Fig. 4. The scale of the UV absorption was adjusted as the amount of conjugated diene isomers in the sample was estimated to be 76% (16). ▲, UV detection; △, EC detection.

In our preceding work (11), the separation of phospholipid hydroperoxides from unoxidized phospholipids was achieved by reverse-phase HPLC using a C-18 column. This column was replaced by a C-8 column to elute the phospholipid hydroperoxides and unoxidized phospholipids in a shorter time without increasing the flow rate during the analysis. Thus, the HPLC condition described here may be more suitable for practical use.

It has been suggested that hydroperoxy phospholipids can be removed by reduction of the hydroperoxy group directly (12) or after release of the hydroperoxy free fatty acids by reaction with phospholipase A_2 (17,18). Hydroxy derivatives may accumulate instead of the hydroperoxides in membrane phospholipids when lipid peroxidation proceeds in vivo. Thus, the specific detection of the hydroperoxy group is required when phospholipid hydroperoxides are determined by reverse-phase HPLC, because phospholipid hydroperoxides and their hydroxy derivatives are not separated from each other. The EC detector seems able to detect hydroperoxides selectively under HPLC analysis. Furthermore, the EC detector in this condition is more sensitive than the UV detector, based on conjugated diene absorption as shown in Figures 6 and 7.

In conclusion, the combination of reverse-phase HPLC and the EC detector can serve as a sensitive and specific method for the quantitative analysis of phospholipid hydroperoxides. The application of this method to lipid peroxidation products in vivo will be reported in a following paper.

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Effects of Parenteral Nutrition with High Doses of Linoleate on the Developing Human Liver and Brain

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The developmental changes in the fatty acid composition of ethanolamine phosphoglycerides (EPG) and choline phosphoglycerides (CPG) were studied in the liver and brain of 18 newborn infants with gestational ages ranging from 20 to 44 wk. A small group of five newborns receiving total parenteral nutrition (TPN) with high doses of linoleic acid (18:2ω6) was also studied and compared to controls of the same gestational age to look for effects on the developmental fatty acid patterns of liver and brain EPG and CPG. TPN with Intralipid 20% was given for 4–12 days, the total fat intake being 14.7–90 g (mean ± S.D. = 47.1 ± 29.8 g). The main developmental changes in the liver and brain of the control group were an increase in 22:6ω3 (docosahexaenoic acid) at the end of gestation and a linear decrease in 20:4ω6 (arachidonic acid) and 18:1ω9 (oleic acid) in EPG and CPG. A very good correlation in the percent values of these fatty acids in the brain and liver tissues was obtained. Very significant changes in the fatty acid composition of liver EPG and CPG could be found in the infants receiving TPN with Intralipid–mainly an increase in 18:2ω6, a decrease in the linoleate elongation/desaturation to longer members of the series and a decrease in the 22:6ω3 levels of liver EPG and CPG. In the brain, only an increase in the 18:2ω6 value of CPG, not accompanied by any increase in the longer ω6 fatty acids, could be detected. Possible adverse effects of high doses of 18:2ω6 on the tissue levels of long chain polyunsaturated fatty acids (PUFA), especially of 22:6ω3, are discussed.
Lipids 22, 133–138 (1987).

Neural tissues are by far the richest in polyunsaturated fatty acids (PUFA), and a remarkable constancy in the fatty acid composition of the brain, despite widely different dietary intakes of essential fatty acids (EFA) has been well documented (1–3). In contrast, the liver is much more readily influenced by differences in the diet (3,4). Dietary EFA are chain elongated and desaturated in the liver to form long chain PUFA, which are preferentially taken up by the brain, especially during development. A progressive increase in the fatty acid length and degree of unsaturation from maternal liver to placenta, fetal liver and fetal brain has been found (5). Direct incorporation of dietary long chain PUFA into the developing brain has also been demonstrated (6).

Besides being influenced by diet, the fatty acid composition of body organs changes with development. Special attention has been focused on developmental changes of the brain fatty acid patterns (7–11), but few data are available on the fatty acid composition of the mammalian liver during development (12,13). Clandinin et al. (14,15) have applied a quantitative approach to the study of total fatty acid accretion in the developing human liver and brain as a means of estimating neonatal dietary needs for ω6 fatty acids but, to our knowledge, a systematic study on the developmental fatty acid patterns of the main phosphoglycerides of human liver and brain is lacking.

For many years it has been general practice in pediatrics to supplement milk formulas with substantial amounts of linoleic acid (18:2ω6). Linoleate supplementation has even been extended to cases in which oral feeding is not possible, and several parenteral formulas highly enriched in 18:2ω6 have appeared. The content and proportion of α-linolenic acid (18:3ω3) have relatively been disregarded in most of these formulas. There is an increasing concern for the metabolic effects of such unphysiological EFA supplies, and some effects on the levels of arachidonic acid (20:4ω6) (16,17), long PUFA with 22 carbon atoms (17,18) and prostaglandin production (19) have been published. However, there is little information on the effects of total parenteral nutrition (TPN) with high doses of 18:2ω6 on the fatty acid composition of human tissues during development (19).

The aim of the present work was twofold: first, to correlate the fatty acid patterns of the main phosphoglycerides in the liver with those in the brain during normal human development and, second, to study the effects of a high supply of linoleic acid, a widely used formula (TPN with Intralipid) administered intravenously, on the developmental fatty acid patterns of human liver and brain. Some preliminary accounts of this work have appeared elsewhere (20,21).

MATERIALS AND METHODS

Developmental study. The developmental study comprised 18 newborn infants with gestational ages between 20 and 44 wk. Gestational ages were determined by maternal history and clinical examination; only those cases with body weights appropriate for gestational age according to Lubchenco's grid (22) were included. Those cases with any kind of neonatal problem that could affect cerebral integrity were excluded. The cause of death, occurring during the first 48 hr of life, was immaturity and/or acute respiratory disease.

Study on TPN. Five infants who received TPN and died during the neonatal period due to major surgical problems were studied. Four were full-term neonates (gestational ages between 37 and 43 wk) and the fifth had been born slightly premature (35 wk). Body weight was appropriate for gestational age in all cases. All infants had to be administered TPN because of multiple congenital defects requiring major surgery (two cases of esophagus atresia and tracheoesophageal fistula, one associated to duodenal obstruction; one case of myelomeningocele plus diaphragmatic hernia and arthrogryposis; one case of intestinal malrotation and duodenal stenosis; and one case of imperforate anus). TPN was infused immediately after operation, performed during the first 24 hr of life. Total fluid intake was 156 ml/kg/day, and total calories ranged from 85.5 to 102 kcal/kg/day. The intravenous infusion

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contained amino acids (1.5–2.5 g/kg/day), glucose (11.2–12.9 g/kg/day), electrolytes and minerals (Na, 2.2 meq/kg/day; K, 1.8 meq/kg/day; Cl, 2.0 meq/kg/day; Ca, 2.5 meq/kg/day; Mg, 0.5 meq/kg/day; P, 2 mmol/kg/day) and vitamins (A, 1000 IU; D, 100 IU; E, 0.5 mg; B_1, 5 mg; B_2, 1.4 mg; B_6, 1.5 mg; C, 50 mg; niacin, 10 mg; pantothenic acid, 2.5 mg). Fat was administered as Intralipid 20% (Vitrum AB, Stockholm, Sweden). A progressive amount of lipids was infused, increasing from 1.0 g to 4.0 g per kg per day in the course of 7 days. The infants died from various complications at 5–13 days after birth, having received TPN for a minimum of 4 days and a maximum of 12. This represented a total fat intake of 14.7–90 g (means ± S.D. = 47.1 ± 29.8 g). According to the analysis of our batch of Intralipid 20%, the total supply of 18:2ω6 ranged from 6.7 g to 41 g (21.5 ± 13.59 g) and that of 18:3ω3 from 1.2 g to 7.3 g (3.8 ± 2.41 g).

All the bodies were refrigerated at 4 C immediately after death. Autopsy, which was done within 12–36 hr of death, did not reveal any brain or liver anomalies. A cerebral hemisphere and the liver were removed, wrapped in double bags of aluminum foil and polyethylene, and stored frozen at −20 C until analysis.

Analytical procedures. All solvents and reagents were of highest purity, purchased from Merck (Darmstadt, Federal Republic of Germany). Just before lipid extraction, the complete hemisphere and the whole liver were homogenized in a Sorvall Omnimixer (Norwalk, Connecticut) to avoid regional differences. Brain and liver homogenates were diluted with an equal volume of water, and total lipids were extracted with 20 vol of chloroform/methanol (1:2, v/v) as specified elsewhere (23). Ethanolamine phosphoglycerides (EPG) and choline phosphoglycerides (CPG) were separated from aliquots of the lower phase by thin layer chromatography (TLC) in two different ways. In order to have a high fatty acid/impurities ratio, as much as 40 μg of lipid P were spotted on a precoated Silica Gel G plate (Chromatoplate, Merck) for the separation of EPG. For CPG, on the other hand, only 20–25 μg of lipid P were spotted on a 0.25-mm-thick Silica Gel G plate prepared manually in the laboratory, because this procedure was shown to give best separation between choline and serine phosphoglycerides. In both cases, the solvent system was chloroform/methanol/water (65:25:3.5, v/v/v). The individual phosphoglycerides were recovered from the plates by scraping off the spots, and the fatty acid methyl esters were obtained directly from the dried silica by cold methanolysis with 2 ml of 0.1 N sodium methoxide in dry methanol, according to Svennerholm (24). After neutralization with 1 N acetic acid, the fatty acid methyl esters were extracted three times with 2 ml of very pure petroleum ether (40–60 C), washed twice with water and dried with anhydrous sodium sulfate. After evaporation with N_2, the fatty acid methyl esters were dissolved in a small volume of hexane and injected into the gas chromatograph.

A Perkin Elmer 900 gas chromatograph was used, equipped with flame ionization detectors (FID) and a 10-ft, 1/8 inch OD, stainless steel column, packed with 15% ethylene glycol succinate (LAC 4R 886) on Chromosorb W 100–120 mesh, acid-washed and dimethyldichlorosilane-treated. The carrier gas was N_2, and the column was operated on a temperature program from 160 C to 195 C, at a rate of 3 C/min. A relatively high volume (10 μl) was injected so that the descending effect of the solvent tail compensated for the column bleeding, and a good base line was obtained despite the use of a single column. The peak areas were measured with an Autolab electronic computer-integrator, system IV model, and the results were expressed as area percentages, which were essentially equivalent to weight percentages within the range of fatty acids studied. The statistical analyses were effected with an electronic programmer (Olivetti, 203 model). For the study on normal development, regression analysis was used, either linear or quadratic depending on the developmental trend. All cases were analyzed individually, with gestational ages plotted on the X axis against corresponding fatty acid values on the Y axis (for correlation between liver and brain, the fatty acid value for the liver was plotted on the X axis against the corresponding value for the brain of the same child on the Y axis, as shown in Figs. 1–3). The levels of significance of the regression coefficients are indicated in Results. On

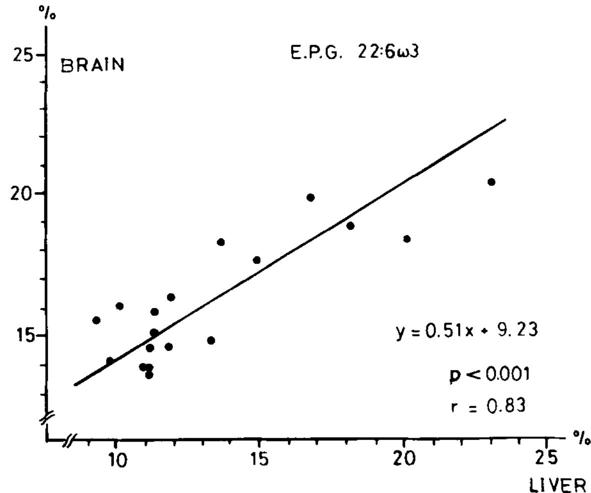

FIG. 1. Brain against liver percent values of docosahexaenoic acid in ethanolamine phosphoglycerides of a group of newborn infants with gestational ages from 20 to 44 wk.

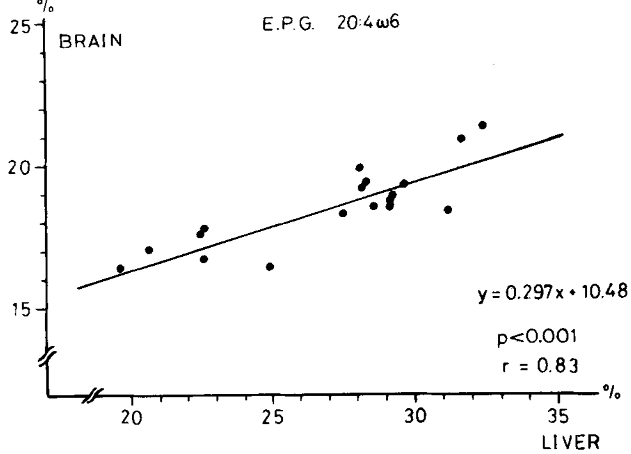

FIG. 2. Brain against liver percent values of arachidonic acid in ethanolamine phosphoglycerides of a group of newborn infants with gestational ages from 20 to 44 wk.

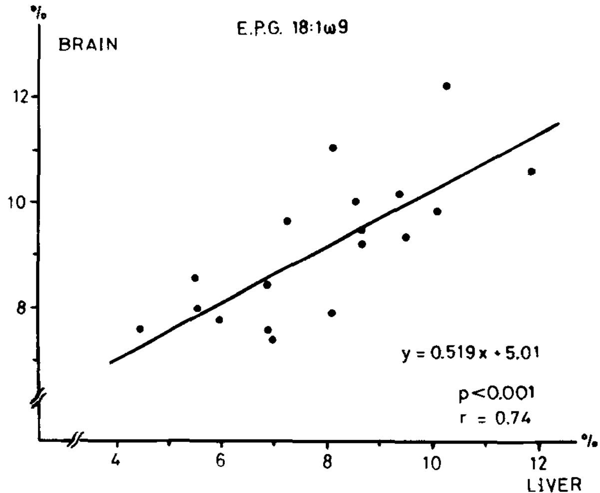

FIG. 3. Brain against liver percent values of oleic acid in ethanolamine phosphoglycerides of a group of newborn infants with gestational ages from 20 to 44 wk.

the other hand, for the study on the effects of TPN on tissue fatty acid composition, all cases were distributed into three age groups (see Results and Tables 1 and 2). This allowed us to compare the cases receiving TPN to controls of the same age. The differences observed were validated by means of Student's t-tests for independent variables, and the levels of significance are given in the tables.

RESULTS

Normal development. Under "controls," Tables 1 and 2 summarize the developmental changes in the fatty acid composition of the main phosphoglycerides of the human liver and brain during the second half of gestation. For the sake of simplicity, Tables 1 and 2 show all the cases distributed into three age groups: a very immature group, ranging from 20–25 wk of gestational age; an immature group in a developmental stage just before the beginning of the "chemical spurt" (23), 28–31 wk; and a full-term group, with gestational ages from 37 wk onward. However, for statistical analysis, all control data were considered individually (by means of regression lines) and not by groups, so that the developmental changes could find a more real statistical expression.

Confirming previous data (10,11), the main developmental changes in the brain were an increase in 22:6ω3 (docosahexaenoic acid) at the end of gestation and a quite linear decrease in 18:1ω9 (oleic acid) and 20:4ω6 in EPG. The elongation/desaturation of arachidonic acid to its products 22:4ω6 (docosatetraenoic or adrenic acid) and 22:5ω6 (docosapentaenoic acid), expressed by the index (22:4ω6 + 22:5ω6)/20:4ω6, increased linearly with gestational age in brain EPG, and the ratio 22:4ω6/22:5ω6 increased with maturation in EPG and CPG as shown before (10). All these changes were very significant statistically ($p < 0.001$).

Very similar changes could be observed in the liver, with 22:6ω3 increasing markedly at the end of gestation in both phosphoglycerides. A linear decrease in 18:1ω9

TABLE 1

Fatty Acid Composition of the Main Phosphoglycerides of the Human Liver—Effect of Development and of Total Parenteral Nutrition (TPN)

	Ethanolamine phosphoglycerides				Choline phosphoglycerides			
	Controls			TPN	Controls			TPN
	20–25 wk (n = 6)	28–31 wk (n = 6)	>36 wk (n = 6)	>36 wk (n = 5)	20–25 wk (n = 6)	28–31 wk (n = 6)	>36 wk (n = 6)	>36 wk (n = 5)
16:0	16.3 ±2.08	16.2 ±1.05	19.3 ±2.94	16.0 ±2.08[a]	35.4 ±2.33	33.7 ±1.06	36.5 ±1.34	32.1 ±1.56[d]
16:1ω7	0.8 ±0.22	0.7 ±0.16	0.8 ±0.22	0.5 ±0.20[b]	4.1 ±0.76	3.3 ±0.38	3.6 ±1.07	2.2 ±0.61[b]
18:0	22.5 ±1.62	24.9 ±1.42	23.6 ±2.55	25.2 ±2.08	11.2 ±0.39	12.3 ±0.79	11.4 ±1.52	11.8 ±1.34
18:1ω9	9.4 ±2.00	8.2 ±0.95	6.2 ±1.31	8.4 ±0.77[c]	21.0 ±2.48	20.4 ±2.62	17.2 ±2.66	15.4 ±0.81
18:2ω6	3.0 ±0.88	4.4 ±0.86	4.9 ±1.64	15.9 ±3.58[f]	5.7 ±1.61	8.7 ±1.28	9.2 ±2.62	24.8 ±2.86[f]
20:3ω9	1.0 ±0.51	0.8 ±0.17	0.2 ±0.15	0.2 ±0.08	0.8 ±0.43	0.6 ±0.12	0.2 ±0.10	tr. —
20:3ω6	1.6 ±0.27	1.8 ±0.39	1.5 ±0.21	0.7 ±0.21[f]	2.1 ±0.39	2.3 ±0.35	2.0 ±0.57	0.9 ±0.33[d]
20:4ω6	29.7 ±1.89	29.1 ±1.19	21.9 ±2.13	21.6 ±3.89	14.7 ±2.67	14.2 ±2.23	13.1 ±1.91	8.8 ±1.93[d]
22:4ω6	2.2 ±1.20	1.8 ±0.74	1.7 ±0.76	1.2 ±0.19	0.3 ±0.08	0.3 ±0.01	0.3 ±0.04	0.2 ±0.05[b]
22:5ω6	1.4 ±0.75	1.0 ±0.22	1.2 ±0.50	0.7 ±0.20[a]	0.4 ±0.13	0.3 ±0.04	0.3 ±0.12	0.2 ±0.07[b]
22:5ω3	0.3 ±0.11	0.3 ±0.06	0.6 ±0.21	0.5 ±0.13	0.1 ±0.04	0.1 ±0.03	0.3 ±0.07	0.2 ±0.05
22:6ω3	11.5 ±1.05	10.6 ±0.96	17.7 ±3.50	9.1 ±2.57[e]	3.3 ±0.61	3.3 ±0.58	5.4 ±1.66	2.7 ±0.69[d]
ω3/ω6	0.31±0.04	0.29±0.03	0.60±0.16	0.25±0.10[e]	0.20±0.02	0.20±0.03	0.23±0.06	0.08±0.03[f]
22:4ω6/22:5ω6	1.60±0.47	1.78±0.46	1.44±0.43	1.74±0.50	0.94±0.18	1.05±0.17	0.87±0.30	1.25±0.37[a]
18:2ω6/18:1ω9	0.35±0.19	0.54±0.10	0.78±0.18	1.88±0.33[f]	0.28±0.10	0.43±0.09	0.54±0.14	1.62±0.26[f]
Elongation/desaturation 18:2ω6*	12.6 ±4.15	7.97±1.51	5.96±1.93	1.59±0.47[f]	3.22±0.65	2.01±0.34	1.79±0.51	0.41±0.11[f]
Elongation/desaturation 20:4ω6**	0.12±0.07	0.09±0.04	0.13±0.06	0.09±0.02	0.05±0.02	0.04±0.01	0.04±0.01	0.04±0.01

All values represent wt % of each fatty acid (mean values, for the number of cases indicated, *n*) ± S.D. The statistically significant changes for the children receiving TPN are signaled by letters according to the following levels of significance (based on Student's t-test with 9 degrees of freedom): *a*, <0.1; *b*, <0.05; *c*, <0.02; *d*, <0.01; *e*, <0.002; *f*, <0.001.

*(20:3ω6 + 20:4ω6 + 22:4ω6 + 22:5ω6)/18:2ω6; **, (22:4ω6 + 22:5ω6)/20:4ω6.

TABLE 2

Fatty Acid Composition of the Main Phosphoglycerides of the Human Forebrain—Effect of Development and of Total Parenteral Nutrition (TPN)

	Ethanolamine phosphoglycerides				Choline phosphoglycerides			
	Controls			TPN	Controls			TPN
	20–25 wk (n = 6)	28–31 wk (n = 6)	>36 wk (n = 6)	>36 wk (n = 5)	20–25 wk (n = 6)	28–31 wk (n = 6)	>36 wk (n = 6)	>36 wk (n = 5)
16:0	7.8 ±0.56	7.6 ±0.66	6.2 ±0.36	5.8±0.56	50.3±0.66	50.6 ±0.71	50.4 ±0.80	49.9 ±0.45
16:1ω7	0.6 ±0.18	0.8 ±0.09	0.5 ±0.12	0.6 ±0.11	7.4 ±0.41	6.7 ±0.50	6.3 ±0.61	6.5 ±0.55
18:0	25.6 ±0.68	27.5 ±1.69	26.7 ±1.49	26.7 ±1.07	7.5 ±0.48	7.9 ±0.50	9.1 ±0.87	8.6 ±0.54
18:1ω9	10.0 ±1.44	9.4 ±1.18	8.0 ±0.41	7.4 ±0.66	23.0 ±0.36	22.6 ±0.88	22.4 ±0.61	21.8 ±0.50
18:2ω6	0.5 ±0.10	0.6 ±0.15	0.5 ±0.12	0.6 ±0.23	0.7 ±0.19	0.8 ±0.18	0.7 ±0.16	1.6 ±0.41[f]
20:3ω9	1.4 ±0.43	1.0 ±0.20	0.8 ±0.31	0.7 ±0.10	0.2 ±0.07	0.2 ±0.07	0.2 ±0.05	0.1 ±0.03
20:3ω6	0.6 ±0.12	0.9 ±0.18	1.1 ±0.17	1.1 ±0.23	0.3 ±0.18	0.5 ±0.06	0.8 ±0.13	0.6 ±0.18
20:4ω6	20.0 ±0.90	18.5 ±0.21	17.0 ±0.58	17.7 ±0.93	4.5 ±0.45	4.7 ±0.32	5.3 ±0.48	5.4 ±0.15
22:4ω6	11.6 ±0.80	13.1 ±1.06	14.8 ±1.02	14.7 ±0.86	0.6 ±0.04	0.7 ±0.04	0.8 ±0.09	0.9 ±0.24
22:5ω6	5.8 ±0.40	6.0 ±0.58	4.6 ±0.85	5.3 ±1.10	0.3 ±0.03	0.3 ±0.04	0.2 ±0.11	0.3 ±0.09
22:5ω3	0.3 ±0.05	0.3 ±0.05	0.5 ±0.21	0.6 ±0.24	tr. —	tr. —	tr. —	tr. —
22:6ω3	15.3 ±0.58	13.8 ±1.09	18.9 ±1.06	18.5 ±1.81	1.1 ±0.11	1.0 ±0.15	1.2 ±0.19	1.4 ±0.23
ω3/ω6	0.41±0.02	0.36±0.03	0.50±0.06	0.49±0.06	0.17±0.02	0.17±0.02	0.18±0.02	0.16±0.03
22:4ω6/22:5ω6	2.01±0.15	2.20±0.11	2.78±0.49	2.84±0.52	2.00±0.13	2.14±0.20	3.51±1.09	2.70±0.45[a]
18:2ω6/18:1ω9	0.05±0.01	0.06±0.01	0.06±0.02	0.08±0.03	0.03±0.01	0.04±0.01	0.03±0.01	0.08±0.02[f]
Elongation/desaturation 18:2ω6*	86.3 ±16.5	73.3 ±19.7	86.9 ±19.2	78.5 ±32.7	8.34±1.56	7.82±1.38	10.8 ±2.21	4.77±1.40
Elongation/desaturation 20:4ω6**	0.88±0.05	1.04±0.09	1.09±0.10	1.13±0.06	0.21±0.03	0.23±0.02	0.20±0.03	0.23±0.05

All values represent wt % of each fatty acid (mean values, for the number of cases indicated, *n*) ± S.D. The statistically significant changes for the children receiving TPN are signaled by letters according to the following levels of significance (based on Student's t-test with 9 degrees of freedom): *a*, <0.1; *f*, <0.001.

*, (20:3ω6 + 20:4ω6 + 22:4ω6 + 22:5ω6)/18:2ω6; **, (22:4ω6 + 22:5ω6)/20:4ω6.

and 20:4ω6 was also found in EPG and CPG, significant at the 0.001 level. The 22:4ω6/22:5ω6 ratio, on the other hand, did not show significant variations with age, these fatty acids being quantitatively less important in the liver than in the brain due to a poorer elongation/desaturation of arachidonate [see the index (22:4ω6 + 22:5ω6)/20:4ω6 in the tables]. As for the parent ω6 fatty acid, 18:2ω6, it is present only in very low amounts in brain because of its very active elongation and desaturation to the higher members of the family. In liver, on the other hand, it is an important constituent of phosphoglycerides, and it increased slowly with maturation in EPG ($p < 0.05$) and CPG ($p < 0.01$), at the same time as its elongation/desaturation slowed down in both phosphoglycerides ($p < 0.001$). As a consequence of these maturational changes, the ratio 18:2ω6/18:1ω9 increased linearly and very significantly ($p < 0.001$) in liver EPG and CPG.

The striking similarity of some fatty acid profiles in the liver and the brain was emphasized by plotting the brain values against the corresponding liver values. This resulted in a very significant ($p < 0.001$) positive correlation between the brain and liver values of 22:6ω3 (Fig. 1), 20:4ω6 (Fig. 2) and 18:1ω9 (Fig. 3). Although only a minor constituent, 20:3ω9 was also studied for its significance in relation to nutrition and showed a parallel decrease with maturation in EPG of liver ($p < 0.001$) and brain ($p < 0.01$) and in liver CPG ($p < 0.001$).

Influence of TPN on fatty acid patterns of liver and brain. The fatty acid composition of liver and brain EPG and CPG in a group of full-term infants receiving TPN with Intralipid is shown in Tables 1 and 2 beside the normal patterns corresponding to controls of the same age, so that the effects of TPN can be easily detected. It can be seen that there was a threefold increase in linoleic acid in the two phosphoglycerides of liver and a twofold increase in brain CPG, all very significant statistically (a t-test was applied between the two full-term groups, control and TPN-treated; see tables for levels of significance). This increase in 18:2ω6, however, was not accompanied by any parallel increase in the long members of the family. On the contrary, 20:3ω6 (dihomo-γ-linolenic acid) decreased very significantly in liver EPG and CPG, and 20:4ω6 decreased markedly in liver CPG (although not in EPG). The longer ω6 PUFA, 22:4ω6 and 22:5ω6, showed a tendency to decrease in liver EPG. It can be deduced, therefore, that the elongation/desaturation of 18:2ω6 was significantly decreased in both phosphoglycerides of the liver, leading mainly to reductions in 20:4ω6 in CPG and in the 22 carbon atom members in EPG.

The ratio of 18:2ω6 to 18:1ω9, which increased slowly with maturation in the liver of the normal child, was much more augmented in the liver phosphoglycerides of infants receiving TPN. Palmitic (16:0) and palmitoleic (16:1ω7) acids were slightly reduced in the liver of the TPN-treated group, more significantly in CPG than in EPG, but 18:0 (stearic acid) did not show any significant variation, and 18:1ω9 slightly increased in liver EPG and showed a nonsignificant tendency to decrease in liver CPG.

A most interesting finding was the very significant decrease in the 22:6ω3 level of the liver, which was reduced to about half the normal value at this age in liver EPG and CPG. As a consequence, the ω3/ω6 ratio was also greatly reduced in the liver of children receiving TPN.

It is interesting to note that the only change in the brain of the parenterally nourished infants was the abovementioned increase in 18:2ω6 in brain CPG. A consequence of this was a very significant reduction of the 18:2ω6 elongation/desaturation and an increase in the 18:2ω6/18:1ω9 ratio in brain CPG. The rest of brain CPG fatty acids, however, were totally within normal limits, and no variation at all could be found in brain EPG.

DISCUSSION

The developmental changes in the fatty acid composition of the main phosphoglycerides in the human brain confirm our previous data on a larger number of cases (10,11), i.e., there is a significant increase in the 22:6ω3 level of the brain EPG and CPG after 32 wk of gestational age and a linear decrease in the 20:4ω6 and 18:1ω9 values during the second half of gestation, mainly in brain EPG.

The present data show an excellent correlation between the main developmental fatty acid changes in the brain and the liver during early human development. In other words, docosahexaenoate increases in the human liver also in a parabolic manner, whereas arachidonate and oleate decrease in a linear way in the two main phosphoglycerides, mainly in EPG. During this crucial stage of brain development the percentage of 22:6ω3 in the liver EPG is almost as high, or even a little higher toward the end of gestation, as that in the brain EPG, indicating that during this period elongation and desaturation of ω3 fatty acids in the human liver is very active and capable of providing the high levels of long ω3 PUFA required by the developing brain.

Upon comparison of fatty acid patterns in the infants receiving TPN with controls of the same age, the decrease in the docosahexaenoate level in both liver phosphoglycerides is striking. Such a decrease in 22:6ω3 has not even been described in rats receiving high doses of 18:2ω6 intravenously (17), in which a similar reduction was noted in liver CPG, but not in liver EPG.

A significant decrease in liver 20:4ω6 has been found in rats (16,17) and humans (19) receiving TPN with high doses of 18:2ω6. In our study, this decrease was only significant in liver CPG, the levels of arachidonate in liver EPG being totally within normal limits for the age. On the other hand, we found a very important increase in 18:2ω6 in both phosphoglycerides of the liver and even in brain CPG, which has not been described in the experimental animal; as a consequence, the ratio of 20:4ω6 to 18:2ω6 (or, as we prefer, the elongation/desaturation of linoleate expressed by the index [20:3ω6 + 20:4ω6 + 22:4ω6 + 22:5ω6]/18:2ω6) was greatly reduced, even much more so than in the rat receiving a large supply of parenteral 18:2ω6 (16).

For many years, studies on EFA have focused mainly on the linoleate (ω6) family. On the other hand, the essentiality of α-linolenic acid (18:3ω3) has been questioned (25), because no signs of ω3 deficiency could be discovered in laboratory animals subjected to ω3-deficient diets. However, from a quantitative point of view, the main long chain PUFA of the ω3 series, docosahexaenoic acid (22:6ω3), is a very important component of neuronal and retinal membranes. Although its functions are still not fully understood, 22:6ω3 seems to be an important factor in membrane fluidity of neurons and outer segments of the retina, probably enhancing the various movements of rhodopsin (26). Docosahexaenoate may even play a role in neurotransmission, as indicated by its preferential accumulation in synaptic membranes (27). Furthermore, another member of the ω3 family, eicosapentaenoic acid (EPA, 20:5ω3), plays an important role in platelet aggregation (28–31), and the supply of ω3 fatty acids is now recommended in the prevention of thromboembolic disorders (32–35).

Recently, clinical evidence of ω3 deficiency, caused by a poor supply of α-linolenic acid, has been presented in the human (36) and in the monkey (37). The present results suggest that a relative deficiency of ω3 fatty acids can also be produced by a different mechanism. It is very possible that the exclusive concern for the ω6 fatty acids and the very high doses of linoleate used in many milk formulas and most forms of TPN has caused the natural preference of the Δ6-desaturase system by the ω3 family (38,39) to be overcome and displaced towards the predominant ω6 fatty acids. Thus, even if a ω3 fatty acid deficiency is spontaneously very rare, if possible at all, because of the abundance of these fatty acids in nature, their small minimum requirements and the enzyme preferences for the linolenate family, our results show that it is possible to artificially produce a relative deficiency in ω3 fatty acids by supplying a great excess of ω6 fatty acids, as has also been shown in the rat brain and retina (40) and liver (17). It seems advised, therefore, to warn clinicians, mainly pediatricians, against the use of excessive amounts of linoleic acid in the diet, especially during early development.

Our cases received TPN only for a short period (4–12 days), and this was enough to produce important alterations in the fatty acid composition of the main liver phospholipids and even a significant increase in 18:2ω6 in brain CPG. The brain's resistance to changing its fatty acid composition despite dietary manipulation is well known (1,2,41,42), and we could not find any alteration in the brain long chain PUFA in our cases receiving TPN. However, it is possible that a more prolonged diet with large doses of 18:2ω6 could affect these fatty acids, as has been shown in the experimental animal (40). In any case, a diet capable of reducing the level of liver EPG and CPG docosahexaenoate to half its normal value during a critical developmental stage should certainly be proscribed. In agreement with very recent data in the rat (17), our results indicate that a correct ratio of linoleate to linolenate is not the only relevant factor in devising an EFA diet, especially when the nutrient has to be administered intravenously. An excess of 18:2ω6, even if a theoretically correct ratio of 18:2ω6 to 18:3ω3 is maintained, should be avoided if the balance of long chain PUFA is to be kept unaltered, an aim particularly important during brain development.

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Time Course of Incorporation of 20-Carbon Polyunsaturated Fatty Acids in a Human Keratinocyte Cell Line

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Human keratinocytes (NCTC 2544) in culture were labeled with equal amounts of either ^{14}C-arachidonic acid, ^{14}C-dihomo-γ-linolenic acid or ^{14}C-eicosapentaenoic acid. At various time points, the incubations were stopped and the distribution of the ^{14}C-fatty acids was analyzed. All these eicosanoid precursor fatty acids were effectively incorporated into the cellular lipids of the keratinocytes, and the major radiolabeled individual lipid fraction was phosphatidylethanolamine. The distributions of arachidonic acid and dihomo-γ-linolenic acid within cellular lipids were rather the same. However, less eicosapentaenoic acid than either arachidonic acid or dihomo-γ-linolenic acid was incorporated into the phospholipids and, correspondingly, more eicosapentaenoic acid was incorporated into the nonphosphorus lipids. In the phosphatidylinositol + phosphatidylserine fraction, there was significantly less eicosapentaenoic acid than either arachidonic acid or dihomo-γ-linolenic acid. The present study suggests that these eicosanoid precursor fatty acids are effectively incorporated into the human keratinocytes and that the pattern of incorporation and distribution of eicosapentaenoic acid appears to differ slightly from that of either arachidonic acid or dihomo-γ-linolenic acid.
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Eicosanoids, derivatives of the 20-carbon polyunsaturated fatty acids (1), play an important role in both physiological and pathophysiological reactions of human skin (2,3). The precursor fatty acids for the eicosanoids are dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid, respectively. The initial step in the formation of eicosanoids is obviously the release of the precursor fatty acids from the cellular lipid stores (4). Dietary factors may affect the formation of eicosanoids by altering the tissue levels of different precursor fatty acids, and, notably, the biological activities of the metabolites derived from each of these fatty acids may differ considerably (5–7). Recently, it has been demonstrated that the distribution and release of arachidonic acid in human keratinocytes may be affected by certain physical and chemical factors (8–10).

When considering the possibilities of dietary alterations in modifying the production of eicosanoids, for example, in inflammatory reactions, one must know if their precursor fatty acids (i.e., dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid) are equally incorporated and distributed in the cellular lipids. In the present study, the time course of incorporation and subsequent distribution of arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid into the lipids of human keratinocytes in culture have been investigated.

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MATERIALS AND METHODS

Chemicals. ^{14}C-Arachidonic acid (sp act 58 mCi/mmol), ^{14}C-dihomo-γ-linolenic acid (sp act 56 mCi/mmol) and ^{14}C-eicosapentaenoic acid (sp act 58.6 mCi/mmol) were purchased from Radiochemical Centre (Amersham, England). Radioactive fatty acids were converted to their sodium salts and added to cell cultures in 100 μl of 0.9% NaCl. Unlabeled reference compounds for the thin layer chromatograms were purchased from Sigma Chemical (St. Louis, Missouri).

Cell culture. In this study a human keratinocyte cell line NCTC 2544 from Flow Laboratories (Rickmansworth, England) was used. This cell line originates from a clone of skin cells derived from a male Caucasian (8). On the basis of both phase contrast microscopy and transmission electron microscopy this cell line has been classified as epithelial (9). Several lines of evidence indicate that the NCTC 2544 cells are nonmalignant: the cells form a monolayer in culture and exhibit growth inhibition, and by light microscopy the nuclei have been found not to appear malignant (9). Moreover, the cells have been found not to grow in soft agar as most malignant cells have been reported to do (9). These cells do not demonstrate complete keratinization, and apparently they are more like the basal than the stratified cells of epidermis. Cytokeratins have been demonstrated using mouse monoclonal anticytokeratin antibodies (DakoPatts, CK-1) in combination with immunofluorescence staining (goat anti-mouse IgG, Vector Laboratories, Burlingame, California). The cells were cultured (40×10^6 cells per flask) in a medium of 40 ml containing 90% RPMI 1640 (KC Biological) and 10% swine serum (Flow Laboratories). In swine serum, arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid comprised approximately 4.5%, 1.5% and 0.5%, respectively, of total fatty acids. At the start of labeling, 360,000 cpm (0.16 μCi, ca. 2.8 nmol) of the ^{14}C-fatty acid was added to the cell culture. After a labeling period of 30 sec, 12 min, 1 hr, 6 hr, 24 hr or 48 hr, respectively, the labeling medium was removed and the amount of unincorporated radioactivity in the medium was measured by liquid scintillation counting. The experiments of 30 sec are referred to as the 0-hr time point. After the labeling, the cells were washed with a small volume of Hank's buffered salt solution and detached from the culture flasks by gentle mechanical rubbing without trypsin. For the following lipid analysis, the cells were transferred from the culture flasks into glass tubes in a small volume of methanol.

Distribution of ^{14}*C-fatty acids in cellular lipids.* The cells were homogenized by vigorous vortexing in 10 ml of chloroform/methanol (2:1, v/v) containing 0.01% of α-tocopherol to prevent oxidation. Homogenates were filtered through glass wool and mixed with 0.2 vol of 0.12 M KCl (13,14). The upper phases were removed and the organic phases were evaporated into dryness. Six ml of acetone saturated with $MgCl_2$ was added to the residue, and the tubes were stored overnight at −20 C. The tubes were centrifuged, and the supernatants (containing the

neutral lipids) were removed. The neutral lipids were thereby separated from the precipitated phospholipids. After acetone was evaporated into dryness, both the neutral lipid and phospholipid fractions were redissolved in chloroform.

Neutral lipid fractions were further analyzed by thin layer chromatography (TLC) (DC-Alufolien Kieselgel 60, Merck 5553) in hexane/diethyl ether/acetic acid (60:40:1, v/v/v) (13,14). Phospholipids were analyzed in a two-dimensional system (13,14). The plates were first developed in chloroform/methanol/13 N ammonia/water (70:30:4:1, v/v/v/v), dried in air flow and developed in the second direction in chloroform/methanol/acetic acid/water (75:45:12:3, v/v/v/v). In this chromatography, phosphatidylinositol (PI) migrated on the same area as phosphatidylserine (PS). For the separation of PI and PS, the corresponding area was scraped from the TLC plate and extracted with chloroform/methanol/acetic acid/water (50:39:1:10, v/v/v/v) (15). The samples were then rechromatographed using a solvent system of chloroform/methanol/acetic acid/formic acid/water (45:10:1:5:1, v/v/v/v/v) (10,16). The lipid fractions were identified by unlabeled standards that were visualized by exposure to iodine vapor. The distribution of radioactivity on the TLC plates was determined by autoradiography of the TLC plates, and the amount of radioactivity on the TLC plates was further determined by liquid scintillation counting.

Statistical analysis. To test the statistical significance of observed differences between fatty acids, the data was following inverse sine (arscine) transformation subjected to two-way analysis of variance. If two-way analysis showed that differences existed, the comparisons of the fatty acids to each other were performed using Bonferroni's test (Tables 1 and 2).

TABLE 1

Statistical Analysis of Data Presented in Figure 1

		PL		NPL	
		20:3	20:5	20:3	20:5
0 hr	20:4	NS	NS	NS	NS
	20:3	—	NS	—	NS
0.2 hr	20:4	NS	NS	NS	NS
	20:3	—	NS	—	NS
1 hr	20:4	NS	NS	NS	NS
	20:3	—	NS	—	NS
6 hr	20:4	**	***	NS	***
	20:3	—	*	—	***
24 hr	20:4	**	***	NS	***
	20:3	—	***	—	***
48 hr	20:4	**	***	NS	***
	20:3	—	**	—	***

Data indicates differences in the time course of the incorporation of radiolabeled arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid, respectively, into phospholipids (PL) and nonphosphorus lipids (NPL). Data was subjected to two-way analysis of variance and the pairwise comparisons of the various fatty acids to each other at the various time points were performed using Bonferroni's test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid.

TABLE 2

Statistical Analysis of Data Presented in Figure 2

		PE		PC		PI + PS		SM		PA		TG		DG		CE	
		20:3	20:5	20:3	20:5	20:3	20:5	20:3	20:5	20:3	20:5	20:3	20:5	20:3	20:5	20:3	20:5
0 hr	20:4	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	20:3	—	NS	—	NS	—	NS	—	NS	—	NS	—	NS	—	NS	—	NS
0.2 hr	20:4	*	NS	NS	NS	NS	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	*
	20:3	—	NS	—	NS	—	**	—	NS	—	NS	—	NS	—	NS	—	NS
1 hr	20:4	***	NS	***	**	NS	***	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
	20:3	—	***	—	NS	—	***	—	NS	—	NS	—	NS	—	NS	—	NS
6 hr	20:4	***	NS	***	*	NS	***	NS	***	NS	NS	**	***	NS	NS	NS	***
	20:3	—	***	—	*	—	***	—	**	—	NS	—	**	—	NS	—	***
24 hr	20:4	***	NS	*	NS	**	***	*	***	NS	NS	*	***	NS	NS	NS	***
	20:3	—	***	—	NS	—	***	—	***	—	NS	—	***	—	NS	—	***
48 hr	20:4	***	NS	NS	NS	**	***	***	NS	NS	***	*	***	NS	NS	*	***
	20:3	—	***	—	NS	—	***	—	***	—	***	—	***	—	NS	—	***

Data indicates differences in the time course of the incorporation of radiolabeled arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid, respectively, into various lipid subclasses in human keratinocytes. The data was subjected to two-way analysis of variance and the pairwise comparisons of the various fatty acids to each other at the various time points were performed using Bonferroni's test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, not significant; 20:3, dihomo-γ-linolenic acid; 20:4, arachidonic acid; 20:5, eicosapentaenoic acid. PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI + PS, phosphatidylinositol + phosphatidylserine; SM, sphingomyelin; PA, phosphatidic acid; TG, triacylglycerols; DG, diacylglycerols; CE, cholesteryl esters.

RESULTS

Radiolabeled arachidonic acid (20:4n-6), dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid (20:5n-3) were effectively incorporated into the cellular lipids of human keratinocytes (NCTC 2544) in culture. The amounts of the ^{14}C-fatty acids (360,000 cpm, 2.8 nmol) incorporated increased continuously up to 24 hr when 87.1 ± 0.4%, 84.2 ± 0.5% and 82.4 ± 0.1% of ^{14}C-arachidonic acid, ^{14}C-dihomo-γ-linolenic acid and ^{14}C-eicosapentaenoic acid, respectively, was taken up by the cells. The bulk of the ^{14}C-fatty acids was recovered in different phospholipids, and at 48 hr the amount incorporated into nonphosphorus lipids was ca. 5%, 5% and 12% for arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid, respectively (Fig. 1). At 6 hr and later, there was more ^{14}C-eicosapentaenoic acid than either ^{14}C-arachidonic acid or ^{14}C-dihomo-γ-linolenic acid within the nonphosphorus lipids. After 6 hr the amount of ^{14}C-arachidonic acid or ^{14}C-dihomo-γ-linolenic acid in the triacylglycerols showed only slight increments, whereas the amount of ^{14}C-eicosapentaenoic acid still showed an increasing tendency at 48 hr.

The major radiolabeled phospholipid fraction was phosphatidylethanolamine (PE), in which ca. 50%, 50% and 40% of arachidonic acid, eicosapentaenoic acid and dihomo-γ-linolenic acid, respectively, were recovered. Two other significant radiolabeled fractions were phosphatidylcholine (PC) and PI + PS. Less ^{14}C-eicosapentaenoic acid than either ^{14}C-arachidonic acid or dihomo-γ-linolenic acid was consistently recovered in the PI + PS fraction. The separation of PI and PS from each other was carried out from the samples of the time point of 24 hr. The rechromatography revealed that of the total of ^{14}C-dihomo-γ-linolenic acid, ^{14}C-arachidonic acid and ^{14}C-eicosapentaenoic acid in the PI + PS fraction, only ca. 10%, 5% and 10%, respectively, were associated with PS.

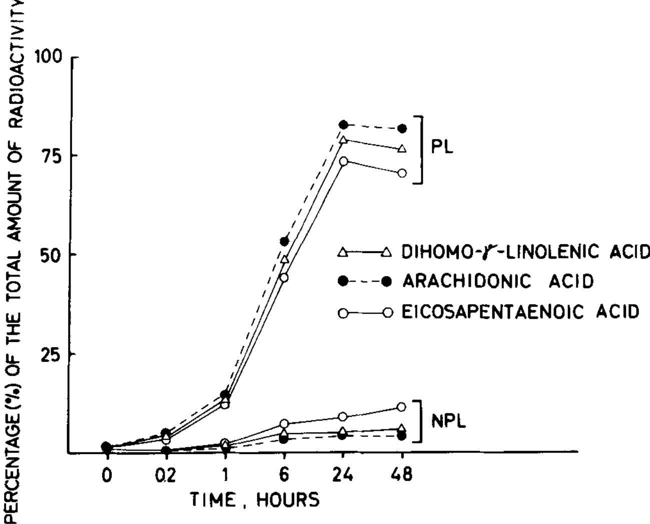

FIG. 1. Time course of the incorporation of radiolabeled arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid, respectively, into phospholipids (PL) and nonphosphorus lipids (NPL) of human keratinocytes in culture. At the time points indicated, the incubations were stopped and the distribution of radiolabel in cellular lipids was analyzed. Values are expressed as percentage of the total amount of radiolabel in each incubation. The values at 0 hr are means of two experiments; values at other time points are means of three (SEM always less than 2%).

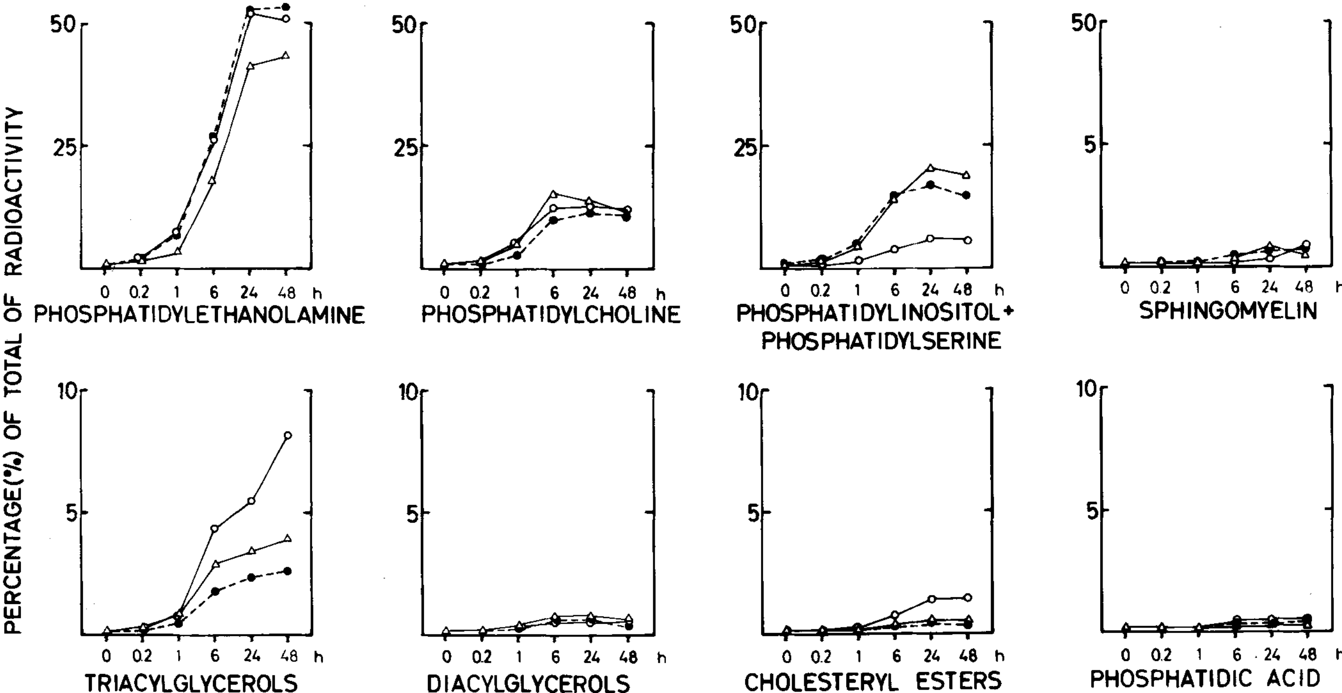

FIG. 2. Time course of the incorporation of radiolabeled arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid, respectively, into various lipids in human keratinocytes. The cells were incubated with 360,000 cpm (ca. 2.8 nmol) of ^{14}C-dihomo-γ-linolenic acid, ^{14}C-arachidonic acid or ^{14}C-eicosapentaenoic acid, respectively. At the time points indicated, the incubations were stopped and the distribution of radiolabel within cellular lipids was analyzed. Results are expressed as percentage of the total amount of radiolabel in each incubation. The values at 0 hr are means of two experiments; values at other time points are means of three (SEM always less than 1%).

Between 6 hr and 24 hr there was a notable increase in the total amount of incorporated radioactivity and this was accompanied by a twofold increase in the amount of radioactivity in PE. After 6 hr the labeling of the PI + PS fraction by either ^{14}C-arachidonic acid or ^{14}C-dihomo-γ-linolenic acid was also increased slightly, but the dominant change in the distribution of the fatty acids was the significant increase in the amount of radioactivity associated with PE (Fig. 2).

DISCUSSION

Arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid were effectively incorporated into the cellular lipids of the human keratinocytes (NCTC 2544). The bulk of the incorporated fatty acids was recovered in various phospholipids. In the nonphosphorus lipids, the amount of eicosapentaenoic acid at either 24 hr or 48 hr was about twofold that of arachidonic acid or dihomo-γ-linolenic acid. The present study, together with previous findings (14), suggests that eicosapentaenoic acid is incorporated less effectively than arachidonic acid or dihomo-γ-linolenic acid into the phospholipids as a whole, and correspondingly more efficiently into the nonphosphorus lipids.

The two major features in the labeling of the phospholipids of keratinocytes were the effective incorporation of all these fatty acids into PE and the poor incorporation of eicosapentaenoic acid into the PI + PS fraction. Since at 24 hr, only minor amounts of radioactivity were recovered in PS, it is apparent that the incorporation of ^{14}C-fatty acids into PI was mainly responsible for the total of ^{14}C-label in the PI + PS fraction. After 6 hr, the labeling of PE showed considerable increments, whereas only slight changes were detected in the labeling of the other phospholipid fractions. This finding is apparently related to the phospholipid composition of the keratinocytes, as PE comprises ca. 40% of the total amount of phospholipids in this cell line (14). It is thus evident that PE has the highest capacity to incorporate eicosanoid precursor fatty acids. The preferential incorporation of arachidonic acid into the PI + PS fraction may be related to the selective acylation of arachidonoyl-CoA or the effective synthesis of arachidonoyl-CoA by specific arachidonoyl-CoA synthetase (17,18). In the present study, the results concerning incorporation and distribution of ^{14}C-arachidonic acid are in relatively good agreement with a previous report on ^{14}C-arachidonic acid incorporation into murine keratinocytes in culture (19). Also with respect to the amount of eicosapentaenoic acid in the PI + PS fraction the present data are fairly consistent with the previous studies (16,20–22), although results of another kind have also been reported (23).

The kinetics and the underlying mechanisms of the acylation of dihomo-γ-linolenic acid and eicosapentaenoic acid into membrane lipids are only poorly understood. In the present study, the patterns of incorporation of arachidonic acid and dihomo-γ-linolenic acid were similar, suggesting that dihomo-γ-linolenic acid could well compete with arachidonic acid for the same binding sites in the cellular lipids. Eicosapentaenoic acid was also effectively incorporated into the keratinocytes, although relatively minor amounts were recovered in the PI + PS fraction. Arachidonic acid and dihomo-γ-linolenic acid thus seem to be the preferential 20-carbon fatty acids in the PI + PS fraction of human keratinocytes. This may be of importance under conditions associated with the stimulated breakdown of inositol-containing phospholipids, which are closely related to the release of eicosanoid precursor fatty acids (23).

Modifications in the dietary fatty acid composition have been shown to alter the fatty acid composition of body tissues; furthermore, at least in some tissues, such modifications have been shown to induce changes in the formation of eicosanoids (6,25,26). The biological activities of the eicosanoids of the 1-, 2- and 3-series may differ considerably. As the eicosanoids of the different series may also interfere with the biosynthesis of other eicosanoids, it is possible that changes in the fatty acid composition of tissues could interfere with the formation of lipid-derived inflammatory mediators (5,27–30). As a conclusion from the present findings, arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid are effectively incorporated into the cellular lipids of human keratinocytes during short-term culture. The practical implications of the present study remain to be elucidated.

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The Formation of Lysophosphatidylinositol Phosphate in Human Platelet Microsomes

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The formation of lysophosphatidylinositol phosphate (lysoPIP) from lysophosphatidylinositol (lysoPI) via kinase activity was studied in microsomal preparations from human platelets. For this purpose, [^{3}H]lysoPI or [^{3}H]phosphatidylinositol ([^{3}H]PI) was prepared and incubated in the presence or absence of ATP, $MgCl_2$ and Triton X-100, and the appearances of radioactivity in [^{3}H]lysoPIP and [^{3}H]phosphatidylinositol phosphate ([^{3}H]PIP), respectively, were monitored using thin layer chromatography. Both lysoPI and PI phosphorylations were completely dependent upon the presence of ATP and $MgCl_2$ in the incubation medium; Triton X-100 addition stimulated both reactions, with the stimulation of PI conversion being considerably greater than that for lysoPI conversion. The present results demonstrate that lysoPI can be converted to lysoPIP by phosphorylation in human platelet microsomes. The potential significance of this enzymatic reaction in stimulated cells is discussed in relation to the generation of inositol-1,4,5-trisphosphate, an important intracellular second messenger.
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Extensive research has been done on the metabolism of polyphosphoinositides. This has included investigations on phosphatidylinositol (PI) and phosphatidylinositol-4-phosphate (PIP) kinase activities as well as the actions of phosphodiesterases and phosphomonoesterases in mammalian cells (1–8). However, by comparison, very little work has been done investigating the metabolism of the lysophosphoinositides. Of the studies performed, most pertain to lysophosphatidylinositol (lysoPI) and its acylation to PI via microsomal acyltransferase activity (9–11); little information is available on the metabolism of the lysopolyphosphoinositides, lysophosphatidylinositol-4-phosphate (lysoPIP) and lysophosphatidylinositol-4,5-bisphosphate (lysoPIP$_2$). With respect to these latter lipids, Palmer (12) has shown that they are not reacylated when incubated with radioactive arachidonic acid, but do undergo a rapid dephosphorylation to lysoPI when incubated with rat brain or liver microsomes. He also concluded that neither the lysopolyphosphoinositides nor the lysoPI derived from them were degraded by acyl hydrolases or phosphodiesterases. Recently, Murase and Okuyama (13) have reported a membrane-bound phospholipase C in porcine platelets with an apparent specificity for lysoPI.

The stimulation of platelets with thrombin, collagen or Ca^{2+} ionophore (14–16) results in the production of lysoPI via the phospholipase A_2-mediated breakdown of PI. Based on previous work in mammalian cells, the fate of the newly formed lysoPI could involve reacylation to PI (9–11), deacylation to glycerophosphorylinositol (17) or degradation by phosphodiesterase activity (13). Since PI is interconvertible with PIP and PIP$_2$ via a phosphorylation-dephosphorylation cycle, it was of interest to determine if another potential metabolic fate might involve the enzymatic phosphorylation of lysoPI to lysoPIP. For this purpose, radiolabeled lysoPI was prepared and incubated with platelet microsomes. Parallel incubations containing radiolabeled PI were conducted for comparison.

MATERIALS AND METHODS

Materials. [2-^{3}H]Glycerol (200 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, Massachusetts). Phospholipase A_2 (*Crotalus adamanteus* snake venom), Triton X-100, adenosine triphosphate (ATP) and lipid standards were purchased from Sigma Chemical Co. (St. Louis, Missouri). Blood collection bags were from Travenol Canada (Mississauga, Ontario, Canada). Precoated thin layer chromatography (TLC) plates (silica gel 60) were purchased from E. Merck, associate of BDH Chemicals Canada Ltd. (Toronto, Ontario, Canada). All chemicals and solvents were of analytical grade. Siliconized glassware was used during the isolation and incubation of platelets.

Preparation of substrates. For the preparation of [^{3}H]PI, blood samples were drawn from the antecubital vein of human donors into blood collection bags containing 63 ml of an anticoagulant solution (2.0 g dextrose, 1.7 g sodium citrate, 206 mg citric acid, 140 mg monobasic sodium phosphate, 17 mg adenine). The blood was centrifuged at 250 × g for 7 min at 22 C. The platelet-rich plasma (PRP) was removed and 100 mM Na_2EDTA was added (10 ml per 150–180 ml PRP obtained from each donor). After the PRP was spun at 2000 × g for 15 min at 22 C, the platelets from each donor were resuspended in 15 ml of platelet-poor plasma and incubated with [2-^{3}H]glycerol (2.0 mCi) for 3.5 hr at 37 C. The tubes were cooled on ice and centrifuged at 2000 × g for 15 min at 4 C. The platelets were then resuspended in a Tris/saline buffer (2 mM Na_2EDTA, 0.15 M NaCl, 0.02 M Tris hydroxy methyl aminomethane, pH 7.4) and recentrifuged at 2000 × g for 15 min at 4 C. The final platelet pellet was resuspended in a glucose buffer (134 mM NaCl, 15 mM Tris hydroxy methyl aminomethane, 5 mM glucose, pH 7.4) before extraction of the lipids by the method of Bligh and Dyer (18). After removal of the lower chloroform phases, the remaining upper phases were reextracted with additional chloroform and the extracts were combined. The lipid extracts were dried under N_2 to smaller volumes, spotted on precoated silica gel 60 plates and developed in chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v/v/v) to separate the individual phospholipids. The plates were sprayed with 2′,7′-dichlorofluorescein in methanol/water (50:50, v/v) and exposed to ammonia vapor followed by acetic acid vapor before lipid bands were viewed under UV light. The [^{3}H]PI band was scraped from the plate and eluted by the method of Arvidson (19). The specific activity was determined by taking aliquots for scintillation counting and gas liquid chromatographic analysis of the derived fatty acid methyl esters in the presence of an internal standard (20).

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Unlabeled PI was obtained from rat liver extract (obtained by the method of Folch et al. [21]) using the above isolation and elution methods.

The [^{3}H]lysoPI was obtained by incubating [^{3}H]PI with a phospholipase A_2 solution. An aliquot of [^{3}H]PI was added to a tube and dried under N_2. After the addition of 15 µl phospholipase A_2 (5 mg/ml of 50 mM Tris-HCl, 5 mM $CaCl_2$, pH 7.4) and 1.5 ml anhydrous ether, the tube was shaken on a metabolic shaker for 4 hr at 22 C. The reaction was stopped by drying under N_2 and adding 100 µl chloroform/methanol (2:1, v/v). The [^{3}H]lysoPI was isolated by the phospholipid TLC method described previously and eluted by a modification of the Bligh and Dyer method (18) as described by Allan and Michell (22). After removal of the chloroform phase, the remaining upper phase was reextracted twice with additional chloroform and the three extracts were combined. The specific activity of the [^{3}H]lysoPI was determined as for [^{3}H]PI. Similar procedures were performed to obtain unlabeled lysoPI from rat liver PI.

Preparation of microsomes. The isolation and washing of platelets from human donors was performed as described above. The final platelet suspension was placed in an ice-cooled container and sonicated at 100 watts three times for 30 sec (with cooling on ice for 15-sec intervals). The sonicated platelets were centrifuged at 3000 × g for 30 min at 4 C to remove the unbroken cells. The supernatant so obtained was spun at 105,000 × g for 60 min at 4 C. The microsomal pellet was homogenized in a sucrose buffer (0.25 M sucrose, 0.01 M Tris hydroxy methyl aminomethane, pH 7.5), quick frozen using acetone and dry ice, and stored at −80 C until needed for the incubations. Protein concentration was determined on the platelet microsomes by the method of Lowry et al. (23), using bovine serum albumin (Calbiochem, La Jolla, CA) as the standard.

Enzyme assays. Unless indicated otherwise in the text, the standard incubation mixture contained 50 mM Tris hydroxy methyl aminomethane (pH 7.4), 25 nmol substrate ([^{3}H]PI or [^{3}H]lysoPI; 50,000 cpm), 3 mg microsomal protein, 2 mM ATP, 30 mM $MgCl_2$ and 0.25% Triton X-100 in a final volume of 3 ml. The substrates were appropriate mixtures of labeled and nonlabeled compounds. [^{3}H]PI or [^{3}H]lysoPI and Triton X-100 in Tris buffer were preincubated for 5 min at 30 C before initiating the reactions by the addition of microsomal protein, ATP and $MgCl_2$. Incubations were normally conducted for 12 min at 30 C, unless otherwise stated, in a shaking water bath. After stopping the reactions with 11.25 ml chloroform/methanol/concentrated HCl (100:200:5, v/v/v), the lipids were extracted as described earlier for [^{3}H]-lysoPI. The lipid extracts were dried under N_2 to 25–30 µl, spotted on precoated silica gel 60 plates and developed in chloroform/methanol/8 M ammonium hydroxide/water (45:35:8:4, v/v/v/v) for the separation of the polyphosphoinositides and their lyso derivatives. The lipid bands were detected as before and scraped into scintillation vials to which 1.5 ml water and 13.5 ml Aquasol-2 (New England Nuclear) were added prior to counting using a Beckman LS7800 scintillation counter (Beckman, Irvine, California). When calculated, the percent conversions of PI and lysoPI to their corresponding phosphorylated products were determined based upon the radioactivities in the substrate regions of the TLC plate from control (zero-time) incubations and the increased radioactivities found in the product regions of the TLC plate from 15-min incubations.

RESULTS

The distributions of radioactivity among the polyphosphoinositides of human platelet microsomes incubated with either [^{3}H]PI or [^{3}H]lysoPI are given in Table 1. With ATP, $MgCl_2$ and Triton X-100 in the incubation medium, the formation of PIP and lysoPIP occurred when PI and lysoPI, respectively, were the substrates. There was an

TABLE 1

Distributions of Radioactivity among Polyphosphoinositides of Human Platelet Microsomes Following Incubation with Either [^{3}H]Phosphatidylinositol or [^{3}H]Lysophosphatidylinositol[a]

		Net change in cpm from control[b]		
Substrate	Phospholipid fraction	+ATP, +$MgCl_2$, +Triton X-100	−ATP, −$MgCl_2$ +Triton X-100	+ATP, +$MgCl_2$, −Triton X-100
[^{3}H]PI	PIP	+3239	−30	+360
	LysoPIP	+259	+4	+11
	PIP_2	−3	−54	+280
	LysoPIP_2	+4	−32	+38
[^{3}H]LysoPI	PIP	+159	−72	−112
	LysoPIP	+808	−41	+368
	PIP_2	−75	−38	−191
	LysoPIP_2	−40	−58	+38

[a]PI, phosphatidylinositol; lysoPI, lysophosphatidylinositol; PIP, phosphatidylinositol phosphate; lysoPIP, lysophosphatidylinositol phosphate; PIP_2, phosphatidylinositol bisphosphate; lysoPIP_2, lysophosphatidylinositol bisphosphate. Each incubation (3 ml) contained 25 nmol substrate, 3 mg microsomal protein and, where indicated, 2 mM ATP, 30 mM $MgCl_2$ and 0.25% Triton X-100. Incubations were performed at 30 C for 15 min. Results are from a single experiment.

[b]A zero-time incubation served as control.

11.1% and 2.7% conversion of PI and lysoPI, respectively, to their phosphorylated products under the specific conditions used. In separate experiments with 25 nmol PI or 22 nmol lysoPI, the conversions to PIP or lysoPIP were 12.3% and 2.4%, respectively (data not shown). These results indicate that PI conversion to PIP was at least four times greater than that of lysoPI to lysoPIP. Both the PI and lysoPI kinase reactions were completely dependent upon the presence of ATP and $MgCl_2$ in the incubation medium. The detergent Triton X-100 exhibited a stimulatory effect on both reactions, as evidenced by the decreased formations of PIP and lysoPIP when Triton X-100 was removed from the medium. The stimulation of PIP formation by Triton X-100 was four times greater than the stimulation of lysoPIP formation. Without Triton X-100 in the incubation medium, there was an equal amount of PIP and lysoPIP formed (0.307 nmol and 0.305 nmol, respectively) from PI and lysoPI.

Figure 1 shows the time course for the synthesis of PIP and lysoPIP by PI kinase and lysoPI kinase activities, respectively. In general, PIP and lysoPIP formations increased steadily with reaction times up to 30 min, with the latter apparently approaching a plateau at 12 min. Based on these results, incubation times of 12 min were used routinely for studying the effect of microsomal protein and substrate concentrations.

The influence of microsomal protein concentration on the formation of PIP and lysoPIP is shown in Figure 2.

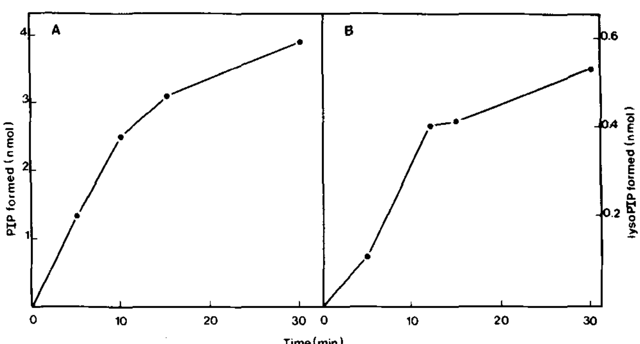

FIG. 1. Effect of incubation time on PIP (A) or lysoPIP (B) synthesis. Standard assay mixtures (as described in Materials and Methods) were incubated for 0–30.

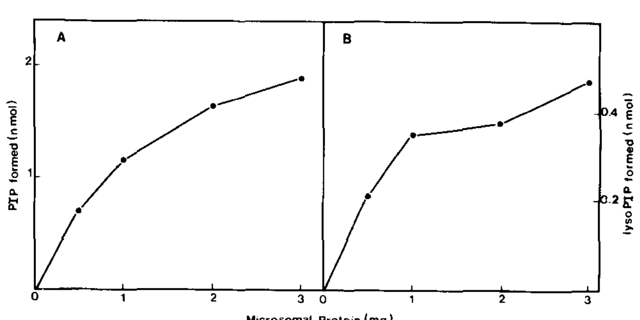

FIG. 2. Effect of microsomal protein concentration on PIP (A) or lysoPIP (B) synthesis. Assay procedures were as described in Materials and Methods except that incubations were performed using 0–3 mg microsomal protein.

The synthesis of PIP and lysoPIP increased progressively with microsomal protein concentrations up to 3 mg. Figure 3 shows the effect of increasing substrate concentration on product formation. There was a linear increase in the production of PIP with increasing PI concentrations, while there was a steady but nonlinear increase in lysoPIP synthesis as lysoPI concentrations increased. Even at the highest substrate concentrations, PIP and lysoPIP formations did not reach a plateau.

DISCUSSION

Previous reports have demonstrated the activity of PI kinase in a number of cell types (1–4). In the present work, we have demonstrated the conversion of lysoPI to lysoPIP in human platelet microsomes via a kinase activity with an obligatory dependency upon ATP and $MgCl_2$. This is of interest since little information is available on the presence of lysoPIP in tissues. In addition, our results indicate the presence of PI kinase activity in platelet microsomes.

The detergent, Triton X-100, exhibited stimulatory effects on the conversions of both PI and lysoPI to their corresponding phosphorylated products (Table 1). This is in agreement with previous work utilizing membranes from liver (24) or plant cells (25) where PIP formation was stimulated by the addition of Triton X-100 and exogenous PI. Without Triton X-100, there was an equal formation of PIP and lysoPIP from their respective substrates, but when Triton X-100 was included in the incubations, PI phosphorylation was stimulated to a much greater extent than lysoPI phosphorylation. This may reflect differences in the solubilities of the substrates, the accessibility to the membrane-bound enzyme and/or the solubilization(s) of enzyme and substrate. It is interesting that upon addition of PI or lysoPI to the microsomal preparation in the absence of Triton X-100, there was a net positive change in the radioactivity associated with the PIP_2 and $lysoPIP_2$ regions, respectively. With Triton X-100 included in the incubation medium, these changes in radioactivity were not observed. Previous studies (3,26) have demonstrated an inhibitory effect of Triton X-100 on PIP phosphorylation to PIP_2. The increased radioactivity in $lysoPIP_2$ in the incubation without Triton X-100 was

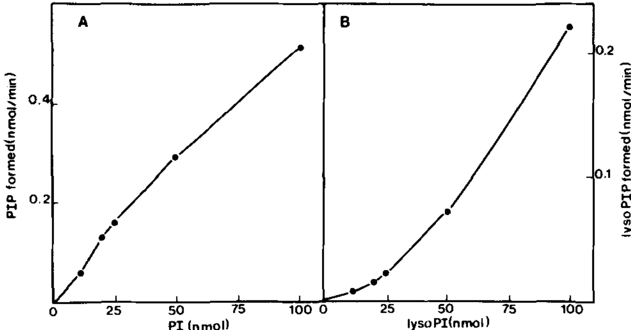

FIG. 3. Effect of substrate concentration on the rate of synthesis of PIP (A) or lysoPIP (B). Assay procedures were as described in Materials and Methods except that incubations were performed using substrate concentrations of 12–100 nmol. Substrates were incubated for 7 or 12 min to determine reaction rates. Zero-time and zero-protein incubations served as controls.

small and insignificant. Whether incubating microsomes with lysoPIP, under conditions favoring PIP_2 synthesis, would result in the formation of $lysoPIP_2$ remains to be determined.

It remains to be studied whether separate kinase enzymes are involved in the phosphorylations of PI and lysoPI. The kinetics of both reactions (Figs. 1-3), although similar, differed in some respects. It may be that the PI kinase enzyme acts on lysoPI as well as PI, but possesses different characteristics toward each substrate. In this regard, Palmer (12) has shown that brain and liver microsomes dephosphorylate the lysopolyphosphoinositides, $lysoPIP_2$ and lysoPIP, and has speculated that the phosphatases responsible are most likely the PIP_2 and PIP phosphatases, respectively. In the case of phospholipase C, separate enzymes appear to act on PI and lysoPI, based upon recent work in porcine platelets (13).

The formation of lysoPIP from lysoPI is a potentially important finding. It has been shown in platelets stimulated with thrombin, collagen or the Ca^{2+} ionophore, A_{23187} (14-16), that the lysoPI level increases, presumably due to a phospholipase A_2-mediated breakdown of PI. From the work reported herein, it can be speculated that some of the newly formed lysoPI could be phosphorylated to lysoPIP. If lysoPIP were formed in intact platelets or mammalian cells, it may be phosphorylated to $lysoPIP_2$ by kinase activity. Subsequent degradation of $lysoPIP_2$ via a phospholipase C activity, analagous to that reported toward lysoPI (13), would result in the formation of monoacylglycerol and inositol-1,4,5-trisphosphate (IP_3). IP_3 has been implicated as a second messenger (27,28) in the mobilization of intracellular Ca^{2+} following agonist stimulation of a variety of cell types, including platelets (29,30), and the subsequent phospholipase C-mediated breakdown of PIP_2. Alternative fates for lysoPIP could include dephosphorylation to lysoPI (12), hydrolysis via phospholipase C to monoacylglycerol and inositol-1,4-bisphosphate, or deacylation/reacylation reaction(s).

In conclusion, we have shown that lysoPI can be converted to lysoPIP by enzymatic phosphorylation in platelet microsomes. The presence and importance of lysoPIP formation in other stimulated and unstimulated mammalian cell types remains to be investigated.

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Effect of Hypothyroidism on the Lipid Composition of Rat Plasma and Erythrocyte Membranes

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The effect of hypothyroidism on plasma and erythrocyte membrane lipid components has been investigated. This pathological state is accompanied by a) a cholesterol increase of about 60% in plasma, and at the same time a 22% reduction in erythrocyte membranes; b) 44% and 30% phospholipid level decreases in both plasma and red cell membranes, respectively; and c) almost unaffected phospholipid and fatty acid compositions of both plasma and erythrocyte membranes. All changes were corrected by treatment of the hypothyroid rats with triiodothyronine for two days. These findings suggest that in hypothyroid rats a reduced transfer of cholesterol from plasma to erythrocyte membrane probably takes place. This could explain, at least in part, the increased hematic cholesterol level observed in hypothyroid animals. In red cell membranes, the simultaneous decrease in cholesterol and phospholipid levels does not alter the cholesterol/phospholipid molar ratio, thus avoiding their abnormal function.
***Lipids 22,* 148–151 (1987).**

Lipids are essential structural components in the membranous structure of cells. The cholesterol to phospholipid (C/P) ratio in the erythrocyte membrane is usually regulated to maintain proper membrane fluidity for normal functioning of the cell. Since erythrocytes have a relatively insignificant degree of cholesterol synthesis (1), the exchange of cholesterol between plasma and erythrocytes is very active both in vivo and in vitro (2). Recently Lange et al. (3,4) have reported a new effect of cholesterol in determining the stability of membrane contour so as to prevent endocytosis.

In biological membranes, phospholipids are asymmetrically arranged, and their exchange between plasma lipoproteins and red cell membranes is very active (5). It has been reported that thyroid hormones affect both plasma cholesterol concentration, hepatic metabolism and cholesterol synthesis (6–8). Recently we have shown the alteration induced by hyperthyroidism on the lipid composition of plasma and erythrocyte membranes (9) as well as on rat liver mitochondria and microsome lipid pattern (10).

The present study was designed to investigate the effects of 6-n-propyl-2-thiouracil (PTU)-induced hypothyroid state on the lipid pattern in both plasma and erythrocyte membranes. The data obtained indicate that in plasma from hypothyroid rats there is an increased cholesterol concentration and a phospholipid decrease. In erythrocyte membranes of the same animals the decrease of cholesterol content, accompanied by a decrease of phospholipids, does not significantly alter the C/P molar ratio. All of the changes were corrected by treatment of the hypothyroid rats with triiodothyronine (T_3) to render them euthyroid. The phospholipid composition as well as the pattern of total fatty acids both in plasma and in erythrocyte membranes is unchanged by propylthiouracil treatment. These results add insight into the role of thyroid hormones in regulating the cholesterol and phospholipid levels in plasma by the active change taking place between plasma and erythrocyte membranes.

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MATERIALS AND METHODS

Animals. Male Wistar rats (200–250 g) fed ad libitum with a standard diet were used and divided into three groups: group 1, normal rats (euthyroid); group 2, rats made hypothyroid by adding PTU (0.1% in tap water) for 3–4 weeks (11) (hypothyroid); group 3, rats made hypothyroid injected intraperitoneally with 3,3′,5-L T_3 (25 μg/100 g body weight) dissolved in 0.9% NaCl/propyleneglycol (40:60, v/v) twice daily to render them euthyroid (treated-hypothyroid) (12). The animals were killed by decapitation, and the blood was collected in a beaker with EDTA as anticoagulant and centrifuged at 750 × g for 7 min. Erythrocytes were washed four times with a buffer containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM NaH_2PO_4, 10 mM Tris and 5 mM glucose, pH 7.4. Hemoglobin-free erythrocyte membranes were prepared according to the method of Dise et al. (13) by hypotonic hemolysis in 80 ml of 10 mM Tris, pH 7.4, isolated by centrifugation and washed four times with the same buffer.

High pressure liquid chromatography (HPLC) analysis of cholesterol, phospholipids and fatty acids. Cholesterol, phospholipids and fatty acids were analyzed by HPLC, using a Beckman 344 gradient liquid chromatography equipped with a Perkin-Elmer LC-55B spectrophotometric detector.

To determine cholesterol (free and total), the chromatographic column was Altex ultrasphere-ODS, reverse phase (4.6 × 250 mm) from Beckman (Palo Alto, California). Free cholesterol was determined in plasma by the method of Duncan et al. (14). The mobile phase was 2-propanol/acetonitrile/water (60:30:10, v/v/v) at a flow rate of 1 ml/min. For total cholesterol determination, both plasma and erythrocyte ghosts were saponified with alcoholic KOH for 60 min at 45 C and extracted with hexane. Then the extract was evaporated and the residue dissolved in 2-propanol, an aliquot of which was injected into the column. The mobile phase was 2-propanol/acetonitrile (50:50, v/v) at a flow rate of 1 ml/min (14).

For fatty acid analysis, both plasma and erythrocyte membranes were saponified for 15 min at 90 C with 5 ml of 5% KOH in 50% aqueous methanol. The solution was then acidified and extracted with chloroform, taken to dryness and esterified with m-methoxy-phenacyl bromide (15). The chromatographic column was Altex ultrasphere-ODS, reverse phase (4.6 × 250 mm) from Beckman. The mobile phase was tetrahydrofuran/acetonitrile/water (45:25:35, v/v/v) at a flow rate of 2 ml/min. For estimation of a single fatty acid concentration, the calculated

peak areas were compared with areas of each fatty acid standard solution.

Total lipids from plasma and erythrocyte membranes were extracted with chloroform/methanol by the procedure of Bligh and Dyer (16). A portion of this extract was digested at 180 C with perchloric acid, after which lipid phosphorus content was determined by the Nakamura method (17). Phospholipids were separated by the HPLC method previously described (10) with an Altex ultrasil-Si column (4.6 × 250 mm) from Beckman. The chromatographic system was programmed for gradient elution using two mobile phases: solvent A, hexane/2-propanol (6:8, v/v) and solvent B, hexane/2-propanol/water (6:8:1.4, v/v/v). The percentage of solvent B in solvent A was increased in 15 min from 0% to 100%. Flow rate was 2 ml/min and detection was at 206 nm. The quantitative estimation of single phospholipid species was carried out by comparing the calculated peak areas with those of each phospholipid standard solution.

RESULTS

The effect of PTU-induced hypothyroidism on plasma free and esterified cholesterol is illustrated in Table 1. The data reported show that both plasma free and esterified cholesterol are increased by about 60% in PTU-treated rats with respect to euthyroid animals. The table also reports plasma concentration of total phospholipids in euthyroid and hypothyroid rats. In the latter, a 44% total phospholipid decrease with respect to euthyroid animals can be observed. The increase of both free and esterified cholesterol and the decrease of phospholipids in hypothyroidism all reverted to normal after two days of T_3 therapy.

Table 2 reports the concentration of total cholesterol and phospholipids in erythrocyte membranes of euthyroid, hypothyroid and treated-hypothyroid rats. From this table, both cholesterol and phospholipids can be seen to decrease by about 22% and 30%, respectively. After T_3 treatment, these changes reverted to normal. It is worth pointing out that the similarity in percentage decrease of both compounds allows the C/P molar ratio to remain almost unchanged in erythrocyte membranes of hypothyroid rats.

Table 3 shows that the individual plasma phospholipids decreased in an approximately parallel manner so that the phospholipid composition was not significantly altered by the hypothyroid state. As observed in plasma, the erythrocyte membrane phospholipid composition is unaffected by PTU treatment.

Lastly, Table 4 reports the mol % distribution of each fatty acid both in plasma and in erythrocyte membranes. It can be seen that the pattern of fatty acids in plasma and in erythrocyte membranes is similar in both euthyroid and hypothyroid rats.

DISCUSSION

It has been shown that administration of PTU to rats reduces the circulating thyroid hormone concentration by about 90% (6). To clarify the influence of the thyroid state on lipid components of plasma and erythrocyte membranes,

TABLE 1

Effect of Propylthiouracil Administration on Cholesterol and Phospholipid Content in Rat Plasma

Animals	Free cholesterol[a]	Increase (%)	Esterified cholesterol[a]	Increase (%)	Phospholipids[a]	Decrease (%)
Euthyroid	0.82 ± 0.12	—	1.85 ± 0.30	—	1.35 ± 0.22	—
Hypothyroid	1.33 ± 0.14[b]	62.2	3.06 ± 0.24[b]	65.4	0.75 ± 0.17[b]	44.4
Treated-hypothyroid	0.85 ± 0.14	—	1.90 ± 0.29	—	1.30 ± 0.20	—

[a]Each value represents the mean ± S.E. obtained for 6 experiments with 5 rats each. Free and esterified cholesterol are expressed as μmol/ml; phospholipids are expressed as μmol lipid Pi/ml. P values vs euthyroid rats were calculated by Student's t-test.
[b]$p < 0.01$.

TABLE 2

Cholesterol and Phospholipid Content in Erythrocyte Membranes

Animals	Cholesterol[a]	Decrease (%)	Phospholipids[a]	Decrease (%)	Ratio cholesterol/ phospholipids
Euthyroid	750 ± 55	—	860 ± 70	—	0.87 ± 0.10
Hypothyroid	585 ± 62[b]	22.0	602 ± 48[b]	30.0	0.97 ± 0.09
Treated-hypothroid	735 ± 48	—	839 ± 51	—	0.88 ± 0.11

The amounts of proteins are similar for both types of rats (4.7–5.0 mg/ml erythrocytes).
[a]Each value represents the mean ± S.E. obtained for 6 experiments with 5 rats each. Cholesterol are expressed as nmol/mg proteins; phospholipids are expressed as nmol lipid Pi/mg proteins.
[b]$p < 0.01$.

TABLE 3

Phospholipid Composition in Plasma and in Erythrocyte Membranes as Determined by High Performance Liquid Chromatography

	Distribution (mol %)[a]			
	Plasma		Erythrocyte membranes	
Phospholipid[b]	Euthyroid	Hypothyroid	Euthyroid	Hypothyroid
PC + LPC	88.8 ± 2.4	88.0 ± 3.0	47.3 ± 2.5	44.9 ± 2.5
PE	4.5 ± 0.5	5.3 ± 0.7	35.0 ± 2.5	34.8 ± 1.3
SPH	6.7 ± 0.9	6.7 ± 0.7	10.5 ± 0.9	11.6 ± 0.7
PS	—	—	7.2 ± 0.8	8.7 ± 0.5

[a]Each value represents the mean obtained for 6 experiments of 5 rats each ± S.E.
[b]PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; SPH, sphingomyelin; PS, phosphatidylserine.

TABLE 4

Pattern of Fatty Acids in Plasma and in Erythrocyte Membranes from Euthyroid and Hypothyroid Rats as Determined by High Performance Liquid Chromatography

	Distribution (mol %)[a]			
	Plasma		Erythrocyte membranes	
Fatty acid	Euthyroid	Hypothyroid	Euthyroid	Hypothyroid
16:0	22.0 ± 1.8	21.4 ± 1.4	31.0 ± 2.0	32.2 ± 1.5
16:1	2.8 ± 0.3	2.0 ± 0.3	2.0 ± 0.2	2.3 ± 0.15
18:0	11.2 ± 0.8	12.4 ± 0.9	13.2 ± 0.8	12.4 ± 1.0
18:1	19.8 ± 1.0	20.7 ± 1.3	14.1 ± 1.3	13.4 ± 1.0
18:2	26.2 ± 2.2	27.0 ± 1.9	12.7 ± 0.9	13.0 ± 1.1
20:4	18:0 ± 1.1	16.5 ± 1.2	27.0 ± 1.6	26.7 ± 1.3

[a]Results are expressed in mol % of the major fatty acids. Values given are means ± S.E. for 6 experiments with 5 rats each.

euthyroid, hypothyroid and treated-hypothyroid rats have been used. In agreement with other authors (6,7), we have found that PTU-induced hypothyroidism enhances rat plasma cholesterol content. On the contrary, in erythrocyte membranes of the same animals, there is a significant decrease of both cholesterol and phospholipid contents. As is well known, the exchange of cholesterol between lipoproteins and erythrocytes is very active both in vivo and in vitro (2,18). Recently we have reported that following T_3 administration to rats, a major cholesterol transfer from plasma to erythrocytes takes place (9). The results reported here confirm our previous hypothesis that the rate of cholesterol transfer from plasma to erythrocytes could depend on, among other factors, circulating thyroid hormone level, i.e., it increases in hyperthyroid rats but is inhibited in the PTU-treated ones. Correction of this cholesterol transfer following two days of thyroid hormone therapy adds further support to this hypothesis.

Another interesting result that emerges from the present work is the decrease in phospholipid content observed both in plasma and in erythrocyte membranes of hypothyroid rats (see Tables 1 and 2). This could probably be attributed to a reduced availability of fatty acids to their decreased hepatic synthesis (19), as well as to an association between the regulation of phospholipid synthesis and the assembly and release of lipoproteins from the liver (20). These data further confirm our earlier studies (9) showing a divergence in the metabolic response to thyroid hormones between phospholipids and cholesterol. A similar difference between plasma cholesterol and triglycerides has also been observed in hypothyroid rats and was found to be connected to a decrease in plasma very low density lipoproteins (VLDL) and to an increase of low density lipoproteins (LDL) and high density lipoproteins (HDL), while intermediate density lipoproteins (IDL) remain constant. Since plasma and erythrocyte phospholipids are in equilibrium, due to the active exchange between the two pools of compounds (22), the lower erythrocyte phospholipid content of hypothyroid rats depends on their reduced plasma concentration.

Distribution of cholesterol within the various lipoprotein classes has been reported by Dory and Roheim (6). These authors have found the greatest increase in cholesterol concentrations in LDL and HDL, although VLDL and IDL also contained higher concentrations,

whereas the decrease in plasma phospholipid concentrations is primarily due to a decrease of phospholipids in HDL fraction (data not reported).

Finally, our results show that an altered circulating thyroid hormone level induces only quantitative changes in the lipids of rat blood. These changes imply that thyroid hormones, or a lack of them, affect both the metabolism process and the lipid distribution.

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Role of the Lymphatic System in the Transport of Absorbed 7,12-Dimethylbenzanthracene in the Rat

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To determine the role of the intestinal lymphatic system in the absorption of a polycyclic aromatic hydrocarbon, 7,12-dimethylbenzanthracene, a radiolabeled preparation of the compound was given by intraduodenal infusion to rats in doses of 10 μg, 10 mg and 20 mg in olive oil solution. The hydrocarbon appeared to be absorbed from the intestine in a fractional manner, ca. 20% of the administered radioactivity being recovered totally in bile and intestinal lymph in 24 hr at all three dose levels. Biliary radiolabel accounted for 75–82% of combined recovery of radioactivity in bile and lymph with all three doses. The recovery of significant amounts of radiolabel in bile before the appearance of isotope in lymph, together with the fact that the biliary radiolabel greatly exceeded at all times the lymphatic recovery of isotope, suggests that an alternative pathway, presumably the portal venous route, is of major importance in the transport of the absorbed hydrocarbon.
Lipids 22, 152–155 (1987).

Polycyclic aromatic hydrocarbons (PAH), composed of three or more fused benzene rings, form a class of highly lipophilic substances of considerable toxicological interest. Among the members of this class are several compounds such as benzo(a)pyrene, 3-methylcholanthrene and 7,12-dimethylbenzanthracene, which on metabolic conversion become potent carcinogens. These compounds are found in significant amounts in certain foods, such as smoked meats and fish (1,2). They are readily absorbed by the gastrointestinal tract, metabolized by phase I and II reactions and excreted mainly by the biliary route as water-soluble forms (3–7). These biliary metabolites are substantially reabsorbed in the intestine and re-excreted; thus, there is a considerable enterohepatic circulation of the metabolites of absorbed PAH (6–8). Because of their high lipophilicity and their administration in lipid meals in studies of absorption in animals, it has been considered that a major route for their transport is as solutes in the liquid triglyceride core of lymph chylomicrons (9–14). However, since the 1960s, the intestine has been known to have a considerable capacity for metabolism of PAH (15), and metabolites formed during the absorptive step are known to be transported in the portal vein (16).

The relative importance of the two routes of transport, portal venous vs lymphatic, for PAH absorbed from the intestine is not known, nor is it known whether the proportions of the absorbed compound transported by these routes vary with the load of compound presented for absorption. The route of transport may be important in the ultimate disposition of these compounds in the body since lymphatic transport affords access to the systemic circulation bypassing the liver.

The present study was undertaken to examine the importance of the lymphatic route in the absorption of 7,12-dimethylbenzanthracene (DMBA) and to determine whether a fixed or variable proportion of the absorbed PAH is transported by the lymphatic route.

MATERIALS AND METHODS

Preparation of test meals. DMBA (98% pure) was obtained from Sigma Chemical (St. Louis, Missouri). (G-^{3}H)-DMBA (43 Ci/mmol; 92.4% pure by thin layer chromatography) was purchased from Amersham Corp. (Oakville, Ontario, Canada). Olive oil, obtained from Diamond Brand Manufacturers (St. John's, Newfoundland, Canada), was found by gas liquid chromatography to be composed primarily of glycerides of oleic (80%), linoleic (9%) and palmitic acids (10%).

Toluene solutions of ^{3}H-DMBA and DMBA were combined in glass vials protected from light and were evaporated to dryness under nitrogen. Olive oil was added to DMBA preparations to achieve final DMBA concentrations of 10 μg (12.5 μCi ^{3}H), 10 mg (12.5 μCi ^{3}H) or 20 mg (12.5 μCi ^{3}H) per ml of oil. Olive oil was chosen as the vehicle since this carrier triglyceride preparation had been used in our previous studies of DMBA absorption (8). The solutions were kept in the dark for 18 hr and examined visually for undissolved hydrocarbon, then were combined with pooled rat bile to yield a final oil to bile ratio of 2:1 (v/v). These mixtures were vortexed to emulsify the preparations, and aliquots of each were taken for liquid scintillation counting. Volumes of 1.5 ml were drawn up into syringes that were weighed prior to and following test meal administration.

Animal surgery. Male Sprague-Dawley rats (275–325 g) were purchased from Charles River Breeding Laboratories (St. Constant, Quebec, Canada) and maintained with free access to food (Purina Rat Chow) and water under standard light and temperature conditions.

After a ventral midline abdominal incision was made under ether anesthesia, the main mesenteric lymphatic duct was exposed and cannulated with vinyl tubing (Dural Plastics and Engineering, Dural N.S.W., Australia; external diameter 0.80 mm, internal diameter 0.50 mm) by the method of Turner and Barrowman (17). The accessory intestinal lymph vessel was obliterated by cutting and application of cyanoacrylate glue to ensure complete collection of intestinal lymph. The common bile duct was cannulated above its confluence with the pancreatic ducts using polyethylene tubing (Clay Adams, Parsippany, New Jersey; PE-10, external diameter 0.61 mm, internal diameter 0.28 mm) and was secured with ligatures of 4 × 0 surgical silk as previously described (8). A saline-filled premature infant nasogastric feeding tube (size 5F, C.R. Bard Ltd., Mississauga, Ontario, Canada) was used to cannulate the duodenum by the method of Laher et al. (8). The lymphatic and biliary catheters were exteriorized through stab wounds in the right flank, while the duodenal catheter was exteriorized through the abdominal wound. The incision was then closed in two layers with silk sutures.

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Immediately following surgery, the animals were placed in restraining cages in a warm room (23 C) for overnight recovery. During this time they were infused intraduodenally with 0.9% NaCl at 2.5 ml/hr. Bile was collected during the first 5 hr and kept under refrigeration. If normal bile and lymph flow, as judged from values obtained in a large series of animals, were not achieved by the end of the 20-hr recovery period, an animal was rejected from the study. Normal flows for lymph and bile were considered to be approximately 1 ml/hr and 0.4 ml/hr, respectively.

Experimental procedure. Thirty-min control samples of bile and lymph were collected into preweighed vials (those for lymph contained sodium citrate to prevent clot formation). Each animal then received a 1.5-ml test meal (containing 10 μg, 10 mg or 20 mg DMBA and 12.5 μCi ^{3}H-DMBA) by intraduodenal infusion at a rate of 1.5 ml/hr. The residual mixture in the catheter was flushed in with a pulse of 0.5 ml bile and followed by an infusion of bile/saline (1:5, v/v) over the next 6 hr at a rate of 4.8 ml/hr. From the beginning of the control period, bile and lymph vials were changed every 30 min, weighed and analyzed for radioactivity. Over the following 17 hr, one collection was performed, during which time an intraduodenal saline infusion of 2.5 ml/hr was given. Following one additional 30-min collection period, the animals were killed by an anesthetic overdose and examined for patency of catheters.

Sample analysis. The volumes of lymph and bile obtained were calculated from their weight using a specific gravity of 1.00 for each. Duplicate 50-μl aliquots of both fluids were mixed with 10 ml Aquasol-2 scintillation cocktail (New England Nuclear, Lachine, Quebec, Canada) and counted in a Beckman LS8100 liquid scintillation counter. Quench was corrected using an external standard.

After removal of samples for analysis, the initial 7-hr lymph collections were pooled from two rats that had received the 20-mg dose of DMBA. The samples were diluted 3:1 with normal saline, and aliquots were removed for scintillation counting. The mixtures were then ultracentrifuged for 30 min in a Beckman 75Ti rotor at 30,000 rpm in a Beckman L2-65B ultracentrifuge. Following gentle aspiration of the creamy lipid layer, samples of the clear infranatant were taken for liquid scintillation counting.

To determine the proportion of radiolabel in the supernatant lipid layer, recovery of hydrocarbon was assessed by liquid scintillation with no differentiation between DMBA or its metabolites.

Statistical analyses were performed using an ANOVA and also Student's t-test for unpaired values where appropriate with significance established to be $p < 0.05$.

RESULTS

Figure 1 shows the appearance of radiolabel in lymph and bile for a 7-hr period following intraduodenal administration of a lipid test meal containing 12.5 μCi ^{3}H-DMBA and 10 μg, 10 mg or 20 mg of DMBA. Little radiolabel could be detected in lymph within 60 min of administration, in contrast to a significant presence of isotope in bile during the same time period. The figure shows the recoveries of radiolabel for each 30-min collection period. In the three groups, bile and lymph flows were similar (results not shown). By 2 hr post-administration, lymphatic radiolabel concentrations increased rapidly, although actual recoveries never exceeded those of bile for any dose of hydrocarbon. The 2-hr lag time in the appearance of significant lymphatic radiolabel coincides with the appreciable appearance of lipid-laden chylomicrons and an increase in lymph volume that follows the administration of fat. Lymphatic transport of tritium began to decline before peak biliary radiolabel was attained with the two smaller doses (Fig. 1). Peak radiolabel concentrations following 10 μg, 10 mg or 20 mg DMBA correspond to 32 ± 6 ng/ml, 36 ± μg/ml and 49 ± 16 μg DMBA equivalents per ml of lymph, respectively, while respective peak biliary radiolabel values represent 271 ± 62 ng/ml, 164 ± 22 μg/ml and 370 ± 80 μg DMBA equivalents per ml of bile.

Figure 2 illustrates the cumulative recovery of radiolabel in lymph and bile from all three doses. The rates of recovery from the lymph (given as a percentage of the

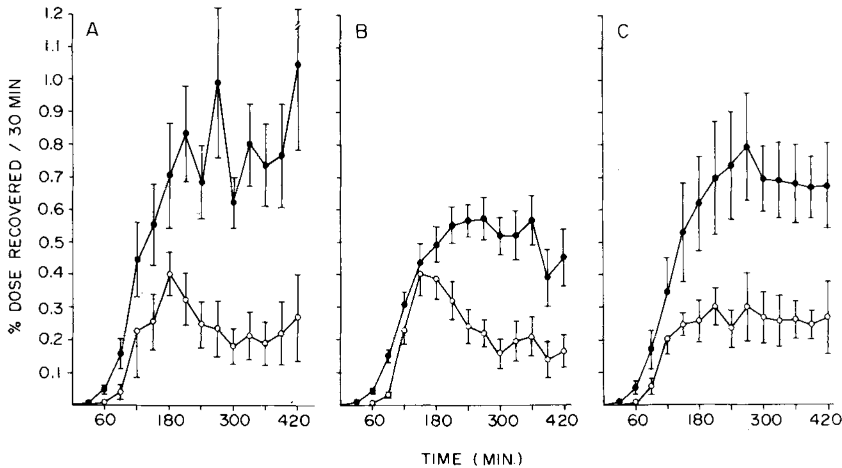

FIG. 1. Total recovery of radiolabel in bile and lymph over 7 hr as a percentage of administered radiolabel (^{3}H-DMBA) for doses of 10 μg (A), 10 mg (B) and 20 mg (C). Closed symbols, bile; open symbols, lymph. Each point represents the mean and SEM for six animals for the 10-μg and 10-mg doses and four animals for the 20-mg dose.

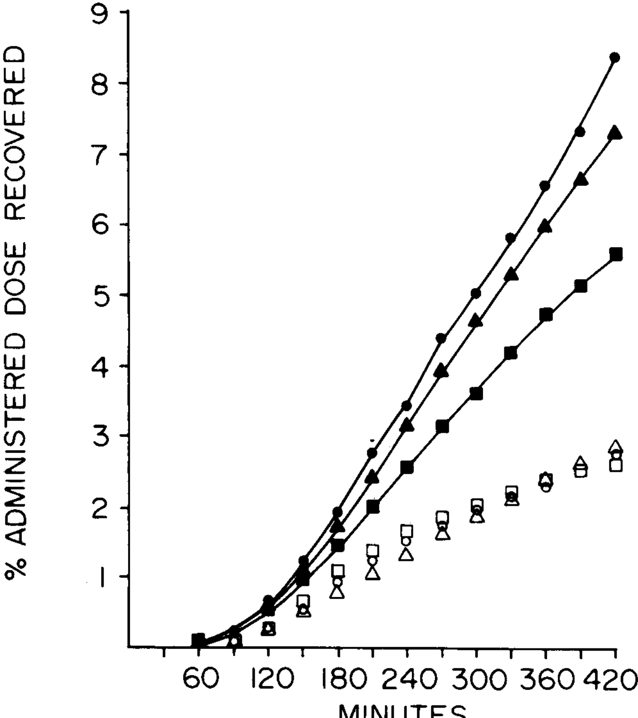

FIG. 2. Cumulative recovery of radiolabel over 7 hr in lymph (open symbols) and bile (closed symbols) for three doses of DMBA. Circles, 10 μg (six animals); squares, 10 mg (six animals); triangles, 20 mg (four animals).

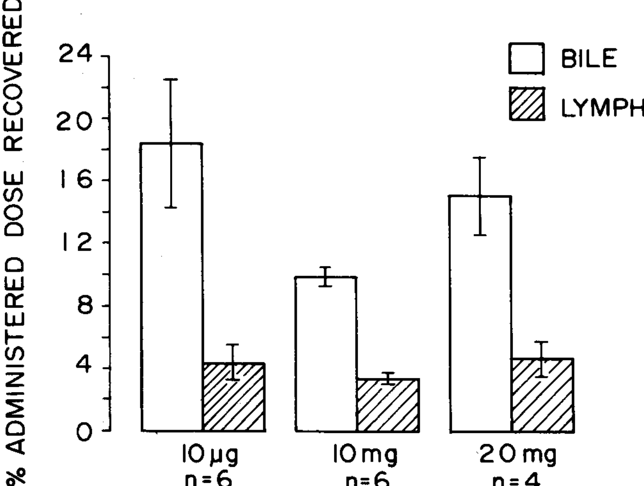

FIG. 3. Total 24-hr recovery of radiolabel in lymph and bile for three doses of DMBA (means and SEM).

administered dose) were nearly identical for all time points throughout the 7-hr study period. Radiolabel appeared in the bile at an approximately constant rate for each dose. These rates exceeded the corresponding lymphatic rates of transport of DMBA. The highest fractional rate of biliary radiolabel recovery occurred with the 10-μg DMBA dose.

The total biliary and lymphatic recovery of radiolabel in the 24 hr following test meal administration are given in Figure 3. Total percentage recoveries were not significantly different among the three doses by analysis of variance ($p > 0.05$), although recovery from the 10-mg dose as compared to the 10-μg and 20-mg doses was somewhat less ($p < 0.01$). Biliary radiolabel accounted for the greatest proportion of retrieved radiolabel, yielding bile to lymph ratios of 4.85 $\pm$ 0.75, 3.05 $\pm$ 0.22 and 3.53 $\pm$ 0.43 for the 10-μg, 10-mg and 20-mg dosages, respectively. The 10-μg dose exhibited a significantly higher bile to lymph ratio and percentage of recovered dose present in the bile than did the 10-mg dose of DMBA ($p < 0.05$), whereas values obtained from the 20-mg dose were not significantly different from either the 10-μg or 10-mg test meals of hydrocarbon ($p > 0.05$).

Significant levels of lymph radioactivity were only obtained once the lymph became turbid with particulate lipid. Following removal of this lipid from pooled lymph by ultracentrifugation, only 14.0 $\pm$ 1.3% of the original radiolabel remained in the infranatant.

DISCUSSION

PAH are lipophilic compounds with very low aqueous solubilities; for example, DMBA has a water solubility of 0.061 mg/l (18). PAH are relatively well absorbed from the gastrointestinal tract when presented in solution in nutrient lipids (9–14) or a solvent such as ethanol (16). Following absorption, DMBA is metabolized in the liver, excreted chiefly in the bile as glucuronide-conjugated metabolites (4,5) and ultimately eliminated in the feces. From the intestine, the compound is partially reabsorbed and reexcreted by the liver (8) in an enterohepatic circulation, which delays its elimination from the body.

There is evidence that following intestinal absorption, PAH are transported in both portal venous blood and lymph, but the proportions of the compounds carried by these two routes are not known. In one recent study of benzo(a)pyrene absorption in sheep, it was shown that the amounts of the compound appearing in systemic blood following intragastric administration in corn oil solution were negligible in animals with intestinal lymph fistulae; this was taken to indicate that lymph is of paramount importance in the transport of absorbed benzo(a)pyrene (14). However, systemic blood concentrations may be an unreliable index of absorption by the portal venous route if hepatic extraction of the compound or its metabolites is efficient. There are many studies that document the appearance of absorbed PAH in intestinal lymph (9–14), and the portal venous transport of metabolites of absorbed PAH is well established. For example, Bock et al. (16), using rat jejunum in situ, found that within 30 min of intraluminal instillation of benzo(a)pyrene dissolved in ethanol, portal blood contained 40% of administered radioactivity; more than 90% was in the form of metabolites.

It is of some importance to know the proportions of the compounds transported via the lymphatic route, since this affords access of the substance to the systemic circulation and extrahepatic tissues. Furthermore, it has been suggested that important interactions may occur between absorbed PAH and lymphoid cells during the passage of these compounds through the lymph and lymph nodes (14). Of interest also is the ultimate disposition of PAH that reach the systemic circulation via the lymph. These compounds might be deposited in peripheral tissues

during lipolysis of chylomicron triglyceride or might be transported to the liver for metabolism and excretion.

The present study was designed to determine what proportion of absorbed DMBA is transported in lymph and whether this is a fixed proportion or is variable and dependent on the mass of compound presented for absorption. The model used is an indirect means of assessing portal venous vs lymphatic transport and takes biliary excretion of metabolites in the presence of complete intestinal lymph diversion as a rough index of portal venous transport. This approach has been used to determine the relative importance of portal venous and lymphatic transport of absorbed nutrient lipids such as retinol in rats (19).

Our data show that over a 2000-fold range of dose of DMBA, the proportions of the compound (or its metabolites) leaving the intestine by the lymphatic route are relatively constant at about 20% of the total amount recovered in lymph and bile. Thus another pathway, presumably portal venous transport, appears to be more important. A recent study has come to a similar conclusion for absorbed benzo(a)pyrene (20). The factors determining the partition between lymph and portal venous blood are presently unknown. It has long been known that the intestinal epithelium can metabolize PAH and that the resulting metabolites are transported in portal venous blood (15,16). In intestinal lymph, the parent compound, DMBA, can be demonstrated as a solute in chylomicrons (unpublished results). The present study, however, has not attempted to determine the chemical form of DMBA or its derivatives in portal venous blood or lymph.

If the predominant form of DMBA in lymph were parent compound and in portal venous blood were in the form of more polar metabolites, one might have expected the compound's partition between portal venous blood and lymph to alter with increasing DMBA loads as the metabolic capacity of the enterocyte was exceeded, but such an effect was not observed in the present experiments. The situation, however, may be very complex, since it is possible that during the hours absorption was being studied the hydrocarbon may have induced the metabolizing enzymes of the enterocyte (21). Another factor remaining to be investigated is the role of the mass of carrier triglyceride, fixed at 1 ml in the present study, in determining the partition of DMBA between lymph and portal venous blood.

Results in the present study suggest that the absorption of DMBA over the large dosage range may be of fractional character, ~20% of the administered radioactivity being recovered in bile and lymph over 24 hr. It is presumed that the 80% unaccounted for represents unabsorbed material. Fractional absorption is observed with certain other lipids, such as sterols. Sylven and Borgström (22) found that an almost constant fraction (about 0.4) of various cholesterol doses, ranging from a trace to 100 μmol, is recovered in thoracic duct lymph. β-Sitosterol is absorbed in a similar fractional fashion (23). The explanation for this type of absorption is not presently known.

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Nonenzymatic Hydrolysis of Phosphatidylcholine Prepared as Liposomes and Mixed Micelles

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Nonenzymatic hydrolysis of phosphatidylcholine was investigated at several pH ranges in liposomes and mixed micelles. Phosphatidylcholine was stable to hydrolysis for 140 hr at pH 4, 7 and 10, but slowly hydrolyzed at pH 1 with a half-time of 50 hr. We conclude that phosphatidylcholine is not significantly degraded by hydrolysis under conditions typical for liposome preparation methods.
Lipids 22, 156–158 (1987).

Although liposomes are increasingly used as model membrane systems in basic and applied research, relatively little is known about their chemical stability. Noncatalyzed hydrolysis of ester bonds linking fatty acyl groups to glycerol would be expected to be fairly slow, yet at least two earlier reports (1,2) have noted a rapid degradation of lipids upon exposure to ultrasonic irradiation. Furthermore, no systematic study has been done to determine long-term chemical stability of liposome in buffered solutions.

Products of degraded lipids such as fatty acids and lysophosphatides can increase bilayer permeability (3). Such alterations may be particularly important for clinical testing of liposome-based therapy (4) and immunoassays, since successful storage of encapsulated material is likely to depend on the stability of lipid.

It is generally agreed that liposomes are sufficiently stable to hydrolysis at neutral pH ranges, so that for most purposes the amount of hydrolysis occurring during preparation and storage times of several hours is negligible. However, in some procedures, lipid may be exposed to aqueous solutions for more than 24–48 hr, for instance, during preparation of liposomes by detergent dialysis (5,6). In other experiments, lipids in the form of planar lipid membranes (7) or liposomes (8) have been exposed to pH values ranging from 1 to 10 for periods of a few minutes to several days.

It is clearly important to know the relative extent of lipid hydrolysis under normal and extreme conditions. To address this question, we have exposed lipid in several physical states (small and large unilamellar liposomes, and mixed micelles in octylglucoside) to different pH ranges and monitored the appearance of hydrolysis products over time. We chose 1-palmitoyl-oleoyl-*sn*-glycerophosphorylcholine as the lipid because phosphatidylcholine is a commonly used lipid in model membrane systems. Furthermore, the presence of specific acyl groups permitted an estimate of the relative rates of hydrolysis at the 1- and 2-positions. Conditions of pH were chosen to represent the extreme values in the literature and included ranges near 1, 4, 7 and 10.

EXPERIMENTAL METHODS

Liposome preparation. 1-Palmitoyl 2-oleoyl phosphatidylcholine (POPC) was purchased from Avanti (Birmingham, Alabama). Large unilamellar vesicles (LUV) were prepared by the methods of Mimms et al. (5). In a typical experiment, 10 μmol POPC was solubilized with 100 μmol octylglucoside in the buffer to a final volume of 10 ml, then passed through a Bio Gel P6 column. Vesicles prepared by this method range around 0.1 μ in diameter. To prepare SUV, the LUV were sonicated in a bath sonicator for 10 min. For lipid in the form of mixed micelles, 10 μmol POPC was solubilized with 100 μmol octylglucoside in the appropriate buffer to a final volume of 10 ml.

We chose to use potassium phosphate solutions for the buffer, because phosphate has useful buffer capacity in the pH ranges used in the study. Stock solutions of the buffer were produced by appropriate mixtures of 0.1 M K_2HPO_4 and phosphoric acid. This provided the same initial conditions for extraction of all samples and placed free fatty acids in their acid (neutral) form to aid extraction. The samples were immediately extracted with 2 ml chloroform/methanol (2:1, v/v) containing myristic acid (50 μg/ml) as an internal standard. The samples were stirred by vortexing, followed by centrifugation (5 min, 1000 × g) to separate the chloroform phase. The upper aqueous phase was carefully removed and discarded, and the chloroform extract was stored at −10 C until analysis.

Before methylation of the free fatty acids, phospholipid was removed from the chloroform extract by filtration through small silicic acid columns (HiFloSil, BioRad) prepared in Pasteur pipettes. The fatty acids were eluted by passing two volumes of chloroform/methanol (1 ml each) through the column, and the eluates were taken to dryness under nitrogen. Methylation was carried out with boron trifluoride/methanol. Methyl esters were extracted from the methylation mixture with 2 ml diethyl ether. One ml of the ether extract was taken to dryness under nitrogen, and 100 μl of carbon disulfide was added to dissolve the esters. One-μl aliquots of the carbon disulfide solution were analyzed by gas liquid chromatography using a 10% SP-2330 Chromosorb column. The amount of each fatty acid present was determined from the ratio of peak areas to the internal standard peak area, followed by comparison with predetermined values to known peak ratios of the fatty acids.

One experimental series was designed to measure appearance of lysophosphatidylcholine (LPC) as a hydrolysis product. In this, POPC liposomes were prepared in 0.1 M K_2SO_4 by octylglucoside dialysis (5), then adjusted to pH 1.0 with 0.1 M phosphate buffer to a final lipid concentration of 1.0 mM. At increasing time intervals, 1-ml aliquots of the suspension were extracted with 2 ml of chloroform/methanol (2:1, v/v), and the chloroform layer was taken to dryness under nitrogen. The lipid was then dissolved in 150 μl of chloroform, and 2 μl was applied to a Chromarod, developed in chloroform/methanol/water (65:25:4, v/v/v) and analyzed with an Iatroscan instrument (9). The amounts of the separated lipid species were determined by comparison of integrated peak areas with known standards of POPC, LPC and fatty acids.

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RESULTS AND DISCUSSION

Effect of pH and physical state on hydrolysis rates. The lipid dispersions were surprisingly stable to hydrolysis in the pH range of 4-10. At the start of the incubation period, ca. 1% of the total fatty acid was present as free fatty acid, and after 140 hr this figure had not significantly increased (Fig. 1). However, significant hydrolysis was observed at pH 1, and about 20% of the total fatty acid ester had been hydrolyzed after 140 hr. The physical state of the lipid did not markedly affect the hydrolysis rate, although there was a trend for lipid dispersed as micelles to be hydrolyzed at a slightly faster rate. Similar hydrolysis rates were also observed at pH 10 with egg phosphatidylethanolamine and bovine brain phosphatidylserine in the form of mixed micelles, LUV and SUV.

Effect of acyl chain position on hydrolysis rates. Figure 2 shows results for relative hydrolysis rates of acyl chains at the *sn*-1 and *sn*-2 positions. Within experimental error, we were unable to distinguish any differences in relative lability to hydrolysis at the two positions. It was possible that LPC was more labile to acid hydrolysis than POPC and that the lack of specificity of hydrolysis rate with respect to acyl chain position was simply due to rapid hydrolysis of LPC to glycerophosphorylcholine. Therefore, the accumulation of LPC at pH 1 was also monitored as described in Methods. We found that as POPC was hydrolyzed, both LPC and fatty acid accumulated at approximately equal rates (Fig. 3). We concluded that the appearance of fatty acid from the *sn*-1 and *sn*-2 positions did, in fact, reflect a nonspecific hydrolysis mechanism.

Comparison with earlier investigations. This report has established that POPC liposomes are reasonably stable to hydrolysis within pH ranges and incubation times (up to six days) used by other investigators. Only near pH 1 was significant hydrolysis observed, and this occurred

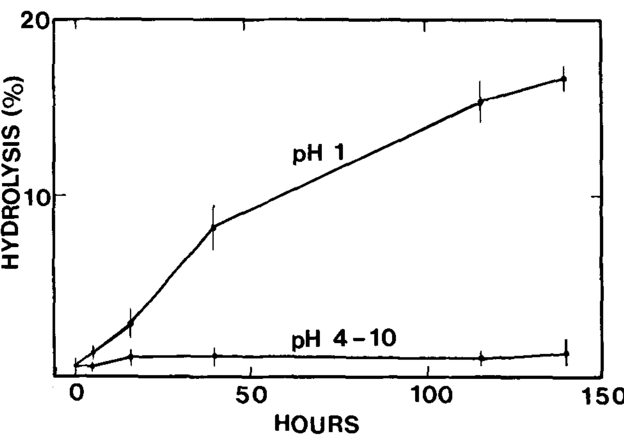

FIG. 1. Dependence of hydrolysis rate on pH. Phosphatidylcholine was dispersed as mixed micelles in octylglucoside and as small and large unilamellar liposomes, as described in Methods. The dispersions were incubated at pH ranges near 1, 4, 7 and 10 for periods up to 140 hr, and hydrolysis was monitored by the appearance of free palmitic and oleic acid in the medium. Since there were no significant differences for hydrolysis rates of lipid in different physical states, the data are summed and presented as mean ± S.D. Little hydrolysis was observed between pH 4 and 10 (lower line). At pH 1, significant hydrolysis occurred, and about 16% of the the total fatty acid was hydrolyzed after 140 hr incubation.

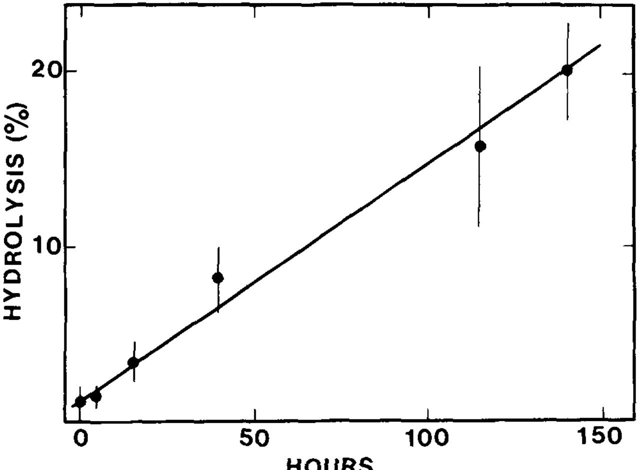

FIG. 2. Hydrolysis rates of palmitic and oleic acid from POPC. Figure shows summed data for palmitic and oleic acid present as mixed micelles of phosphatidylcholine, large unilamellar liposomes and small unilamellar liposomes at pH 1. There were no significant differences for hydrolysis rates of palmitic and oleic acid in the *sn*-1 and *sn*-2 positions, respectively, nor for physical state of the phosphatidylcholine, so each data point represents the mean ± S.D. of six different experiments. The data is presented as percentage of hydrolysis of each fatty acid, rather than as percentage of total fatty acid, as in Fig. 1.

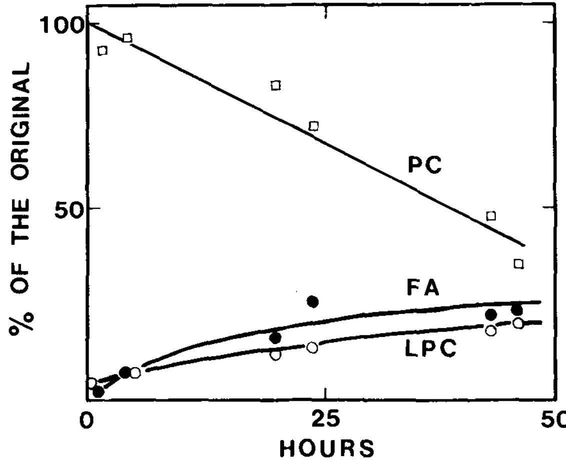

FIG. 3. Rates of POPC disappearance and appearance of LPCFA. In one experiment, both phosphatidylcholine and hydrolysis products were monitored during hydrolysis at pH 1. As phosphatidylcholine was hydrolyzed, fatty acids and lysophosphatidylcholine (LPC) appeared in the medium at approximately 1:1 stoichiometries, suggesting that LPC was not significantly more labile to hydrolysis than phosphatidylcholine.

randomly at the *sn*-1 and *sn*-2 positions. The hydrolysis rate was not markedly affected by the physical state of the lipid at this pH, and small and large unilamellar vesicles, as well as lipid dispersed as mixed micelles in octylglucoside, underwent hydrolysis at similar rates.

Two earlier studies reported results for nonenzymatic hydrolysis of phospholipid. These were not directed at establishing chemical stability of lipid dispersions, but rather to study reaction mechanisms. For instance, Wells (10) and Kensil and Dennis (11) used highly alkaline

conditions to study the effect of aggregation state on hydrolysis rates. In the latter report, lipid was dispersed as monomers, small and large unilamellar vesicles, multilamellar vesicles and mixed micelles in Triton X-100. The lipids were then subjected to alkaline hydrolysis near pH 12.7 (0.132 M NaOH) and the hydrolysis products were analyzed by thin layer chromatography. Possible positional specificity of hydrolysis was also determined by using dipalmitoyl phosphatidylcholine labeled with ^{14}C-acyl chains in the *sn*-2 position. Acid hydrolysis of phospholipid has also been reported (for a review see ref. 12).

Our results for acyl position hydrolysis agree with those of Kensil and Dennis, who observed no significant differences in rates of hydrolysis at the *sn*-1 and *sn*-2 positions. These investigators did find that the alkaline hydrolysis rate in lipid dispersed as micelles was seven- to 11-fold faster than in vesicles, whereas our results for acid hydrolysis did not show this effect. Finally, the rate constants for alkaline hydrolysis were much higher than those for acid hydrolysis in the pH 1 range. For instance, Kensil and Dennis reported $K_1 = 1.4 \times 10^{-3} s^{-1} M^{-1}$, for hydrolysis of egg PC in vesicles at pH 12.7, while our results for hydrolysis of POPC in the range of pH 1 is 5.3×10^{-6}, about 300-fold slower.

In summary, we conclude that phosphatidylcholine is sufficiently stable in the pH range of 4–10 so that liposome preparations and experiments carried out over time intervals of a few hours to several days should not produce significant hydrolytic degradation. At extreme pH ranges in the range of 1 and 12, hydrolysis can occur at significant rates, with half-times from 20 min (Kensil and Dennis) to 50 hr (this report). It is possible that other phospholipids have different hydrolysis rates under certain conditions; this remains to be tested.

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Platelet Changes after a Saturated Fat Meal and Their Prevention by Dazmegrel, a Thromboxane Synthetase Inhibitor

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A saturated fat meal was given to 10 normal males both before and after pretreatment with UK38,485, a thromboxane synthetase inhibitor (Dazmegrel, Pfizer Central Research, Sandwich, Kent, United Kingdom). Following the fat meal at 2 and 4 hr there was laboratory evidence of platelet activation. Dazmegrel lowered baseline platelet activity and lessened platelet activation following the fat meal. This drug should be investigated further as a potential antithrombotic agent.
Lipids 22, 159-162 (1987).

Epidemiological studies (1,2) indicate that a diet rich in saturated fats has a distinct negative influence on coronary artery disease, whereas a polyunsaturated diet might have protective effects (3). In other conditions associated with thrombosis and arterial disease, platelet behavior is influenced by alterations in dietary lipids (4), and it has been well documented that alimentary hyperlipidemia alone may create platelet activation (5-7). That this may be linked to clinical disease is suggested by the work of Regan et al. (8) and Kuo and Joyner (9), who demonstrated decreased oxygen delivery to the myocardium and an increased frequency of angina attacks in fat-fed patients with ischemic heart disease. The mechanism by which a saturated fat diet produces this platelet activation is thought to be related to an alteration in prostanoid production, in particular an increase in production of thromboxane A_2 (TxA_2), the potent platelet aggregant and vasoconstrictor (10). If the effects on platelets are mediated through an increase in TxA_2, it might be reasonable to suggest that its selective inhibition might be an alternative for normalizing platelet behavior in these hyperlipidemic patients.

Dazmegrel (Pfizer Central Research, Sandwich, Kent, United Kingdom) is an imidazole derivative that has been shown to inhibit thromboxane synthetase (11). In addition, this drug may preserve prostacyclin (PGI_2) production (11,12), whose antiplatelet vasodilator effects should further protect against thrombosis.

The aim of our study was to investigate the effects of a saturated fat meal on platelet behavior before and after pretreatment with Dazmegrel. As there is also some work suggesting that acute fat feeding may decrease blood fibrinolysis (13,14), this was also studied.

SUBJECTS AND METHODS

Ten healthy male volunteers (mean age 28 ± 8 yr) were enrolled after informed consent was obtained. Permission for the study was obtained from the regional Ethical Committee. Subjects were fasted for 12 hr overnight and, after baseline blood samples had been taken, ingested 150 ml of double cream (fatty acid content g/100 g cream, shown in Table 1). Further blood tests were taken by separate venipuncture 2 and 4 hr later and after an overnight fast, at 24 hr. Following a one-month washout period, the procedure was repeated. On each occasion, the subjects were pretreated with either placebo or 100 mg of Dazmegrel at 24, 12 and 1 hr prior to the ingestion of cream. The dose of Dazmegrel used has been shown to decrease significantly thromboxane production (12).

TABLE 1

Fatty Acid Composition of Double Cream

Saturated	28.8 g/100 g cream
Monounsaturated	15.4 g/100 g cream
Polyunsaturated	1.3 g/100 g cream

Reference 6.

Blood tests: platelet aggregation (PA). Nine ml of blood was anticoagulated with 1 ml (3.8%) trisodium citrate, and PA was carried out using the conventional aggregating agents adenosine 5′-diphosphate (ADP) and collagen. Two techniques were used: in the first, PA in platelet-rich plasma (PRP) to 2 μm ADP and 2 μg/ml collagen was measured using the established turbidometric technique (15) (Malins photometric aggregometer). Plasma becomes turbid after a fat meal; as this interferes with the light transmission used with PRP aggregation, studies at 2 and 4 hr using this method were not carried out. In the second, PA in whole blood at 37 C was estimated using the Ultraflo 100 Whole Blood Platelet Counter (Clay Adams). To estimate whole blood PA the single platelet count is measured prior to the addition of the aggregating agents (2 μm ADP and 0.5 μg/ml collagen) and again at peak fall in platelet count (1 min for collagen, 3 min for ADP). The initial platelet count will fall as the platelets aggregate. This can be recorded as a percentage of baseline. If the drug protects the platelets against the aggregating agents, then the percentage fall from baseline is less.

Platelet count and volume, white cell count. The Ultraflo whole blood platelet counter was used to measure the number of single platelets. Counts were carried out immediately, the sample was rotated for 5 min at 37 C and the count was repeated. This measures the rate of spontaneous aggregation of platelets with time. A Coulter S Plus was used to measure platelet volume and white cell numbers.

Fibrinogen and fibrinolysis. Nine ml of blood was anticoagulated with 1 ml of 3.8 trisodium citrate. Measurements were made of plasma fibrinogen (16), plasminogen (17) and plasminogen activator (fibrin plate) (18).

Cholesterol and triglyceride. Serum lipids were measured in the routine biochemistry laboratory.

RESULTS

Baseline. When one looks at the two fasting baseline results, the first without pretreatment with Dazmegrel and the second after 100 mg × 3, it can be seen that PA

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in whole blood after addition of both ADP and collagen is significantly decreased after pretreatment with Dazmegrel, although there is no change in the PA in PRP (Table 2). Neither is there any alteration in the platelet count, although the drug does appear to protect the platelets against spontaneous aggregation over 5 min. There is no effect on plasma fibrinogen or fibrinolysis.

Post-fat feeding. When one looks at the effects of fat feeding on the tests, it can be seen that there is a significant increase in triglyceride at 2 and 4 hr (Table 3) but no change in PA in whole blood. There is a significant decrease in platelet count at 2 hr that is not seen after pretreatment with Dazmegrel. Interestingly, the platelet count significantly increases again by 4 hr, a change also abolished by Dazmegrel. There is also a significant increase in platelet volume and total white cell count at both 2 and 4 hr, changes that again are not seen after pretreatment with the drug (Table 4).

Plasma fibrinogen and plasminogen were unchanged by the fat meal, although plasminogen activator activity was significantly increased at 2 and 4 hr with and without Dazmegrel.

DISCUSSION

In this study we have shown that treatment with Dazmegrel significantly protects the platelets against spontaneous PA and against PA induced by ADP and collagen in whole blood. PA to ADP and collagen in PRP, however, was not altered by Dazmegrel. It is possible that there is an increase in production of antiaggregatory prostaglandins from the white blood cells present in the whole blood aggregation system (12). This may explain the discrepancy between whole blood aggregation and PA in PRP. Other thromboxane synthetase drugs have also failed to lower dramatically baseline PRP aggregation

TABLE 2

Platelet Aggregation Pre-Fat Meal and 2, 4 and 24 Hr Post-Fat Meal, Both Before and After Treatment with Dazmegrel (Meal ± SD)

	Pre-meal	2 Hr post-meal	4 Hr post-meal	24 Hr post-meal
Whole blood (%)				
PA ADP				
−UK38,485	43 ± 32	51 ± 35	52 ± 30	38 ± 23
+UK38,485	74 ± 14*	78 ± 21	66 ± 28	69 ± 22
PA collagen				
−UK38,485	13 ± 3	15 ± 11	22 ± 20	25 ± 14†
+UK38,485	19 ± 6††	15 ± 7	23 ± 14	32 ± 28
PRP (% change optical density/sec)				
PA ADP				
−UK38,485	20 ± 6	—	—	19 ± 5
+UK38,485	21 ± 4	—	—	19 ± 4
PA collagen				
−UK38,485	23 ± 7	—	—	24 ± 7
+UK38,485	24 ± 13	—	—	19 ± 6
Spontaneous PA (%)				
−UK38,485	86 ± 9	87 ± 11	89 ± 17	88 ± 12
+UK38,485	95 ± 4*	96 ± 10	95 ± 5	92 ± 9

PA, platelet aggregation; ADP, adenosine 5′-diphosphate; PRP, platelet-rich plasma. *, $p < 0.04$; **, $p < 0.03$; †, $p < 0.02$; ††, $p < 0.007$ (Student's t-test, paired t-test).

TABLE 3

Serum Triglyceride and Cholesterol, Pre-Fat Meal and 2, 4 and 24 Hr Post-Fat Meal, Both Before and After Treatment with Dazmegrel (Mean ± SD)

	Pre-meal	2 Hr post-meal	4 Hr post-meal	24 Hr post-meal
Triglyceride (mmol/l)				
−UK38,485	1.1 ± 0.1	2.0 ± 0.1*	2.7 ± 1.6*	1.0 ± 0.1
+UK38,485	1.1 ± 0.1	2.0 ± 0.1*	3.1 ± 1.9*	1.0 ± 0.1
Cholesterol (mmol/l)				
−UK38,485	5.9 ± 1.0	5.8 ± 1.0*	5.8 ± 1.0*	5.7 ± 1.3
+UK38,485	5.9 ± 1.3	6.0 ± 1.3*	6.0 ± 1.4*	5.8 ± 1.3

*, $p < 0.001$ (paired t-test).

TABLE 4

Platelet Count and Volume, White Cell Count and Plasminogen Activator Activity Pre-Fat Meal and 2, 4 and 24 Hr Post-Fat Meal, Both Before and After Treatment with Dazmegrel (Mean ± SD)

	Pre-meal	2 Hr post-meal	4 Hr post-meal	24 Hr post-meal
Platelet count (× 10^9/l)				
−UK38,485	225 ± 52	201 ± 46	248 ± 38*	206 ± 59
+UK38,485	257 ± 50	256 ± 50	252 ± 53	261 ± 38
Platelet volume (fl)				
−UK38,485	8.7 ± 0.8	8.9 ± 1.0**	9.0 ± 1.0**	8.8 ± 0.9
+UK38,485	8.7 ± 0.8	8.8 ± 0.9	8.9 ± 1.0	8.9 ± 1.1
WBC (× 10^9/l)				
−UK38,485	5.4 ± 0.9	5.8 ± 0.8*	6.2 ± 0.8†	5.1 ± 0.8
+UK38,485	5.7 ± 0.8	5.9 ± 0.6	5.6 ± 1.4	5.4 ± 1.0
Plasminogen activator activity (%)				
−UK38,485	75 ± 17	85 ± 24*	85 ± 17**	72 ± 16
+UK38,485	66 ± 10	82 ± 21†	94 ± 16†	72 ± 9

*, $p < 0.05$; **, $p < 0.03$; †, $p < 0.007$ (paired t-tests).

(19), and it may be that the antiplatelet effects of a drug may not be fully appreciated if work is carried out only in PRP.

Dazmegrel in the dose given does not appear to affect fibrinogen or the measurements of fibrinolysis carried out in this study.

When one looks at the effects of fat feeding on the platelets it can be seen that there is a degree of activation as shown by the fall in platelet count (6) and the increase in platelet volume (7). These changes, however, did not occur after pretreatment with Dazmegrel, demonstrating further protection by the drug.

It was interesting that, despite a fall in platelet count and an increase in platelet volume, PA was unchanged. However, this does agree with the work of Moolten et al. (20) and Bohl et al. (21), who also looked at normal volunteers. In previous work suggesting platelet activation in normal volunteers after a fat meal, two groups used circulating platelet aggregates as their measure of activation (5,6) and platelet stickiness to glass beads (20), and these may be more sensitive tests in this situation. Only animal studies have shown an increase in rate of PA but larger equivalent doses of fat were used in these experiments (22).

We have also shown in this study a significant increase in white blood cell count 2 and 4 hr post-fat meal. The reason for this is not clear, as ingestion of 1.2 l of milk failed to increase either total or differential counts in humans (23). That it could be due to stress is a possibility; however, the subjects experienced no discomfort following the meal. In a previous experiment in our laboratory (24), in subjects smoking three cigarettes in 20 min, which certainly appeared more stressful, the white cell count did not change. Obviously more work is required in this field to determine what types of cell increase and if their behavior is altered. This is of interest, as it is becoming generally accepted that white cells may be more important in the process of thrombosis than was previously thought. Interestingly, whatever the mechanism of the increased white cell count, it seems to have been inhibited by pretreatment with Dazmegrel.

Finally, we could demonstrate no decrease in fibrinolysis using the tests described in this study. This is in contrast to the work of Dubber et al. (13), who studied Europeans, but agrees with Ferguson et al. (14), who looked at an African population. Indeed we detected an increase in plasminogen activator activity, which probably reflects a change from the fasting to nonfasting state. Again it may be of interest to investigate the role of the white cell in this context. However, to assess accurately the effects of fat on fibrinolysis, a nonfat meal should be given as control.

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Biokinetics of and Discrimination Between Dietary *RRR*- and *SRR*-α-Tocopherols in the Male Rat[1]

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The net rates of uptake of the natural (2*R*,4′*R*,8′*R*) diastereoisomer of α-tocopherol (α-T) and the biodiscrimination relative to its 2*S*-epimer (2*S*,4′*R*,8′*R*) have been measured, in two experiments, for the blood and 21 tissues of male Sprague-Dawley rats fed over a period of several months diets containing deuterium-substituted forms of the α-T acetates. Gas chromatography–mass spectrometry was used to measure the amount of deuterated tocopherols taken up relative to the amount of nondeuterated tocopherol remaining. The measurements were performed at different times after the rats, placed for one month on a basal diet containing nondeuterated, natural α-T acetate, were switched to a diet containing the same total quantity of deuterated forms of either natural α-T acetate or a mixture of the acetates of the 2*R*- and 2*S*-epimers (i.e., *ambo*-α-T acetate). In experiment 1 the source of vitamin E in the replacement diet was trideuterio-2*R*,4′*R*,8′*R*-α-T acetate. The data obtained provide the first direct measure of the rate at which natural vitamin E is replaced and augmented in the tissues of growing animals under normal laboratory dietary conditions. There are dramatic differences in the tissue kinetics; for example, the apparent half-life of vitamin E, i.e., the time at which the total amount of ingested trideuterio-α-T taken up is the same as the amount of nondeuterated α-T remaining, varies from ca. 1 wk for the lung to ca. 11 wk for the spinal cord. In experiment 2 the vitamin E in the replacement diet was an equimolar mixture of trideuterio-2*S*,4′*R*,8′*R*- and hexadeuterio-2*R*,4′*R*,8′*R*-α-T acetates. The results show that there is a preferential uptake of the natural diastereoisomer of α-T by all tissues (except the liver during the first month). Examination of fecal material reveals that the biodiscrimination begins in the gut; the incomplete hydrolysis of the acetates shows clearly that this reaction proceeds to a greater extent with the natural diastereoisomer. The greatest discrimination of all the tissues examined was found to occur in the brain. After five months, the level of the deuterated natural diastereoisomer was more than five times that of the deuterated 2*S*-epimer. These results have potential implications for human nutrition.
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Vitamin E appears to owe its bioactivity mainly or entirely to its ability to inhibit lipid peroxidation in vivo (1). This it does by "trapping" the chain-carrying lipid peroxyl radicals, thus "breaking" the free-radical chain process (for concise reviews of the mechanism of lipid peroxidation and its prevention by antioxidants, see refs. 2 and 3). The major component of natural vitamin E, 2*R*,4′*R*,8′*R*-α-T (*RRR*-α-T; Scheme 1) has long been known to show in various animal bioassays a greater activity than synthetic 2*RS*,4′*RS*,8′*RS*-α-T (*all-rac*-α-T), which is an equimolar mixture of all of the eight possible stereoisomers (1).

More recently, it has been shown that the acetates of each of the eight diastereoisomers of α-T all have different activities in the rat fetal gestation-resorption assay, with the natural diastereoisomer, i.e., *RRR*-α-T, being the most active form (4). The differences in biopotencies of the α-T diastereoisomers appear to originate largely from the difference in the chirality at carbon atom 2 (5–10). Thus, the four diastereoisomers with the 2*R* configuration are generally more active than their corresponding 2*S* epimers (in which the CH_3 group and $C_{16}H_{33}$ phytyl group at position 2 are interchanged). Also, single dose experiments in which radioactively labeled 2*R*,4′*RS*,8′*RS*-α-T and 2*S*,4′*RS*,8′*RS*-α-T (or *RRR*-α-T and 2*S*,4′*R*,8′*R*-α-T [*SRR*-α-T]) have been administered to rats (11,12) and chicks (13,14) show that the uptake of the 2*R*-diastereoisomers into tissues is greater than for the 2*S* compounds, strongly suggesting that this is the reason for their greater bioactivity.

We have conducted the first experiments that measure the long-term uptake under normal laboratory dietary conditions of deuterium-substituted *RRR*-α-T and *SRR*-α-T into rat tissues, either singly (*RRR*-α-T only) or competitively (i.e., *RRR*-α-T vs *SRR*-α-T), using diets in which the acetates of these compounds were the only source of vitamin E.

MATERIALS AND METHODS

Materials. [5-CD_3]-2*R*,4′*R*,8′*R*-α-T (d_3-*RRR*-α-T) and [5,7-$(CD_3)_2$]-2*R*,4′*R*,8′*R*-α-T (d_6-*RRR*-α-T) were prepared by the $SnCl_2$-catalyzed deuteriomethylation with perdeutero-

HO, H_3C, CH_3, 5, 7, 2, O_1, 4′, 8′, H, CH_3 — *RRR*-α-T

SCHEME 1

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paraformaldehyde of natural (2R,4'R,8'R) γ- and δ-tocopherols, respectively (15). [5-CD_3]-2S,4'R,8'R-α-T (d_3-SRR-α-T) was obtained after repeated recrystallization of an approximate 80:20 mixture of SRR- and RRR-[5-CD_3]-α-T-p-phenylazobenzoates produced by the $ZnCl_2$-catalyzed cyclization of [5-CD_3]-α-T quinol (16). The quinol was obtained by the $NaBH_4$ reduction of [5-CD_3]-α-T quinone, which in turn was obtained by the $FeCl_3$-catalyzed oxidation (17) of deuteriomethylated 2R,4'R,8'R-γ-T (15). Natural γ-T and δ-T were obtained from soybean oil concentrate. The chiral purity of the deuterated RRR- and SRR-tocopherols was confirmed by measurement of the optical rotation of the potassium ferricyanide oxidation product mixture (16). Tocopherol acetates were prepared by reaction with sodium acetate in acetic anhydride.

Methods. Two experiments were conducted. In both experiments, nine 3-wk-old male Sprague-Dawley rats bred at the NRCC specific pathogen-free facility and housed in individual wire cages were placed for four weeks on a standard AIN-76 diet (major components: 50% sucrose, 20% casein, 15% cornstarch and 5% tocopherol-stripped corn oil) (18) that contained extra menadione [10 times the level in the original diet (19,20)] and 36 mg of 2R,4'R,8'R-α-T acetate (d_0-RRR-α-T-Ac) per kg of diet. After four weeks, the rats were switched to diets in which the (nondeuterated) α-T was replaced by a nominally equivalent amount of either 36 mg of d_3-RRR-α-T acetate (d_3-RRR-α-T-Ac; experiment 1) or a combination of 18 mg of d_6-RRR-α-T acetate (d_6-RRR-α-T-Ac) and 18 mg of d_3-SRR-α-T acetate (d_3-SRR-α-T-Ac) per kg of diet (experiment 2; this combination of diastereoisomers is known as *ambo*-α-T). (All three diets were prepared at NRCC and the tocopherols were incorporated—along with the recommended amount [18] of butylated hydroxytoluene—into each diet after dissolving them in tocopherol-stripped corn oil.)

Blood samples and tissues were obtained from animals after 1, 2, 4, 8, 16, 31–32 and 64–65 days (and 154 days in experiment 2) on the diets containing the deuterated tocopherols.

The blood samples (1 ml), obtained by heart puncture with animals under halothane anesthesia, were withdrawn from one to seven animals (fasted for at least 4 hr) on each of the specified days. The blood was placed in microcentrifuge tubes coated with disodium ethylenediaminetetraacetate and spun at 2000 rpm for 3/4 min in an Eppendorf microcentrifuge. The plasma was removed and stored frozen at −50 C. The red blood cells (RBC) were washed by resuspending them in ice-cold phosphate-buffered saline (5 mM, pH 8.0), spinning for 30 sec, removing the supernatant and repeating the whole procedure twice. The washed RBC were resuspended in the same buffer containing fresh sodium ascorbate (1 mg per ml of buffer) and stored frozen at −50 C. (The ascorbate was added to protect the α-T from oxidation by hemoglobin-derived iron during storage, thawing and extraction.)

Rapid extractions of α-T into heptane were performed on the plasma immediately after thawing by the usual ethanol/heptane procedure (21,22). Typically, 200 μl of ethanol was added to and mixed with 200 μl of plasma followed by 250 μl of heptane. The heptane extracts were stored at −50 C as 2 × 100 μl samples for the purpose of performing replicate analyses.

RBC were extracted using a modified version of the sodium dodecyl sulfate (SDS) method (22). Although volumes and hematocrits varied with each sample, the average volume of RBC suspension in the phosphate/saline/ascorbate buffer used in each extraction was 700 μl, and the average hematocrit was 25% (i.e., 175 μl of packed RBC). The following is an example of a typical extraction: the RBC suspension (700 μl), thawed in ice water, was transferred to a 30-ml glass centrifuge tube. The vial that contained the frozen cells was rinsed with 300 μl of the phosphate/saline/ascorbate buffer, and the rinsings were added to the RBC suspension in the centrifuge tube. SDS (0.1 M; 1.8 ml; 10 × volume of packed RBC) was added to the RBC and mixed briefly on a vortex stirrer, followed by absolute ethanol (2.8 ml, equal to the total aqueous volume), which was also briefly vortex-stirred. n-Heptane (1.0 ml) was added, and the mixture was vortex-stirred for 1 min and centrifuged for 1–2 min. A large fraction (0.8 ml) of the heptane extract was carefully removed and concentrated down under a stream of nitrogen to a volume of less than 200 μl.

Tissues were obtained from rats that had been killed either by decapitation (experiment 1) or by perfusion of saline into the anesthetized animals (experiment 2). The effect of perfusion of the tissues upon the measured ratios of deuterated and nondeuterated tocopherols was determined by using both methods on the two rats that were killed on day 4 in each experiment. All tissues were stored at −50 C. Vitamin E was extracted from aqueous homogenates of the tissues using the SDS method (22). The concentration of the SDS solution used was 0.1 M unless noted otherwise.

All extracts (including those from plasma and RBC) were purified, and the total α-T was quantitated by high performance liquid chromatography (HPLC) (Varian model 5000) using a Lichrosorb Si 60 column (5 μ; Merck, Darmstadt, Federal Republic of Germany) eluted with hexane/t-butylmethyl ether (3%)/2-propanol (0.05%) at 2 ml min^{-1}. 2,2,5,7,8-Pentamethyl-6-hydroxychroman was used as the internal standard (22). Often, the collected fraction containing the α-T was purified further by a second "pass" through the HPLC after being concentrated under a stream of nitrogen. Some modifications of the extraction and purification procedures were necessary depending on the type and amount of the particular tissue.

Liver, lung, kidney, heart, brain, testes, small intestine (duodenum), biceps femoris (1–2 g of each) and spleen (ca. 0.75 g) were homogenized in 6 ml of water (2 × 15 sec using a Brinkmann/Kinematic Polytron PT 10/35 equipped with an anaerobic generator). (In light of the known differences in uptake of vitamin E by different regions of the rat brain [23], the whole brain was homogenized.) A carefully measured volume (2.0 ml) of the homogenate was placed in a 30-ml glass centrifuge tube. SDS (2.0 ml), ethanol (4.0 ml) and n-heptane (2.5 ml) were each added to the homogenate in succession and vortex-mixed briefly (except n-heptane, which was mixed for 1 min). The mixture was centrifuged (1–2 min), and 2 × 1.0 ml of the heptane extract was carefully removed and placed in separate vials. One sample was concentrated to ca. 200 μl under a stream of nitrogen and injected onto the HPLC column after the addition of the internal standard. The other sample was stored at −50 C for a replicate analysis later, if desired.

Epididymal, inguinal and retroperitoneal white adipose tissue (1–2 g of each) were homogenized in a mixture of water (5.5 ml) and SDS (0.5 ml). SDS (1.5 ml), ethanol (6.0 ml) and *n*-heptane (2.5 ml) were each added in succession to the homogenate (4.5 ml) with vortex-mixing in the manner already described. The heptane extract was divided into two 1.0 ml samples. Preliminary HPLC analysis indicated a lot of interference in the region of the α-T peak. Therefore, the sample was subjected to an alkaline hydrolysis to reduce the possibility of interference by ion fragments from other species in the subsequent gas chromatography–mass spectrometry (GC-MS) analysis of the isotopically substituted tocopherols. The sample (1.0 ml) was placed in a 15-ml glass tube equipped with a teflon-lined screw cap, blown down to dryness under a stream of nitrogen and redissolved in ethanol (2 ml) containing sodium ascorbate (1%). Anhydrous sodium methoxide (0.5 M, 4 ml) was then added and the mixture heated at 80 C for 20 min. After the mixture cooled, glacial acetic acid (200 μl) was added followed by water (6 ml) and *n*-heptane (2.5 ml). The mixture was then vortex-stirred for 1 min. A portion (2.0 ml) of the heptane extract was removed, concentrated down to 200 μl under a stream of nitrogen and injected onto the HPLC column. The α-T fraction was collected and blown down, and the residue was subjected to a second alkaline hydrolysis. Again, the heptane extract was passed through the HPLC column and the α-T fraction collected and concentrated.

Interscapular brown adipose tissue (0.4 g) was treated essentially in the same manner as white adipose tissue. Homogenization was performed in water (4 ml), and the entire homogenate was extracted with SDS (2 ml), ethanol (6 ml) and *n*-heptane (2.5 ml). The extract (1 ml) was hydrolyzed and purified twice by HPLC using the method already described.

Diaphragm, soleus, tensor fascia latae and tibialis anterior muscle tissues (0.2–0.6 g) were cut into small pieces with scissors, homogenized in a mixture of water (3 ml) and SDS (0.5 ml, 20 sec) and extracted in the usual manner by adding a further amount of SDS (0.5 ml) followed by ethanol (4 ml) and *n*-heptane (2.5 ml). A portion (1 ml) of the recovered heptane extract (2.0 ml) was purified by HPLC in the usual way.

Aorta (40 mg) was homogenized with a mixture of water (0.5 ml) and SDS (0.2 ml). The probe/generator of the homogenizer was rinsed with water (ca. 1.3 ml) to bring the final aqueous volume to 2 ml. The entire homogenate was treated in the usual way with ethanol (2 ml) and *n*-heptane (2 ml), and the heptane extract (1.8 ml) was purified by HPLC in the normal manner.

Skin (0.4–0.7 g) was cut into small pieces and converted to a powder by cooling with liquid nitrogen and grinding the mixture in a stainless steel mortar and pestle. The powder was homogenized first with water (5 ml, 10 sec) and then with added SDS (0.5 ml, 10 sec). The entire homogenate was extracted in the usual way by adding more SDS (0.5 ml) followed by ethanol (6 ml) and *n*-heptane (2.5 ml). A fraction (1.0 ml) of the recovered extract (2.0 ml) was purified by HPLC.

Fecal pellets (ca. 0.5 g), recovered from the lower part of the large intestine, were homogenized with water (4 ml). A sample of the homogenate (2 ml) was extracted with SDS (2 ml), ethanol (4 ml) and *n*-heptane (2.5 ml). A portion (1.0 ml) of the recovered extract (2.0 ml) was injected onto the HPLC column. The α-T fraction was collected as well as an earlier eluting fraction containing α-T acetate. The latter fraction was blown down to dryness under a stream of nitrogen and the α-T acetate hydrolyzed by heating the residue at 70 C for 30 min with sodium methoxide (0.5 M) in methanol (2 ml) plus methanol (1 ml) containing sodium ascorbate (1%). Glacial acetic acid (100 μl) was added to the cooled mixture followed by SDS (2 ml) and *n*-heptane (2 ml), which were vortex-mixed. The heptane extract recovered after centrifugation was injected onto the HPLC column and the α-T fraction was recovered.

The content and composition of α-T acetate in each diet were checked after saponification of the food to extract the α-T. The diet (0.5 g) was made into a slurry with water (0.5 ml) and warmed at 70 C for 2 min, with a 2% solution of sodium ascorbate in ethanol (2 ml). Saturated aqueous potassium hydroxide (1 ml) and ethanol (1.5 ml) were added, and the mixture was heated at 70 C for 40 min. Water (2 ml) and hexane (6 ml) were added to the cooled mixture and vortex-stirred. A sample (1 ml) of the hexane layer that separated after centrifugation of the mixture for 2 min was removed and concentrated down to 200 μl under a stream of nitrogen (after the addition of an internal standard). The α-T in the concentrated sample was then simultaneously quantitated and purified for GC-MS by the usual HPLC method.

The relative proportions of d_0-, d_3- and d_6-α-Ts were determined by GC-MS of their trimethylsilyl ethers (24). Although the free tocopherols could be successfully analyzed by GC-MS, the trimethylsilyl ether derivatives were used because there was no "tailing" of the peaks. This feature was considered desirable for obtaining more accurate peak area integrations.

The tocopherol trimethylsilyl ethers were prepared by evaporating the purified extracts of α-T in heptane down to dryness under a stream of nitrogen, adding pyridine (100 μl) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) (50 μl; Pierce, Rockford, Illinois) and heating at 65 C for 15 min. After cooling, the mixture was blown down to a small volume (ca. 20 μl). A sample (2 μl) was injected onto a Hewlett Packard Ultra 1 fused silica capillary column (10 m × 0.2 mm ID, OV-101 type, cross-linked, bonded phase) maintained at 275 C in a Hewlett Packard 5790A Series GC, which was connected to a Hewlett Packard 5970A Series Mass Selective Detector set up to monitor continuously the 502 (d_0), 505 (d_3) and 508 (d_6) molecular ions. The peak area data for the 505 and 508 ions were corrected for the contribution (2.37%) from the M+3 ions originating from d_0-α-T and d_3-α-T, respectively.

RESULTS

Table 1 indicates the high degree of purity of the deuterium-substituted α-T. It is clear that by using three mass units separation to distinguish the different α-Ts, the M+3 contributions to the next highest molecular ion are very small and in good agreement with the contribution (2.37%) expected from natural abundance isotopes.

GC-MS analysis of α-T extracted from the saponified diets containing deuterated tocopherols confirmed the absence of the nondeuterated form in each diet and verified that the d_6-*RRR*-α-T-Ac/d_3-*SRR*-α-T-Ac ratio was 1.0 in the diet used in experiment 2.

TABLE 1

Relative Ion Intensities, Measured as Peak Areas by Single Ion Monitoring, of the 502, 505 and 508 Ions Obtained from the Gas Chromatography–Mass Spectrometry of the Trimethylsilyl Ethers of d_0-α-T, d_3-*RRR*-α-T, d_3-*SRR*-α-T and d_6-*RRR*-α-T[a]

Compound	502	505	508
d_0-*RRR*-α-T	100.00	2.25	<0.01
d_3-*RRR*-α-T	1.30	100.00	2.17
d_3-*SRR*-α-T	0.26	100.00	2.22
d_6-*RRR*-α-T	0.13	1.70	100.00

[a]The ion intensities for each compound are expressed as a percentage of the parent molecular ion, which is arbitrarily set at 100%.

TABLE 2

Body Weights (g) of Rats at Time of Death for Tissue Analysis

Day[a]	Experiment 1	Experiment 2
1	260	264
2	293	250
4	281	277
4	282	279
8	308	292
16	365	336
31	417	—
32	—	347
64	—	480
65	485	—
65	459	—
154	—	499

[a]Number of days after 7-wk-old rats were switched to diet containing deuterated α-tocopherol acetate.

Table 2 shows for each experiment the weights of the rats at the time they were killed for tissue analysis.

We will use the symbol ∅ to describe the ratio of total deuterated α-T [$\Sigma(d_x$-α-T); x = 3,6] to nondeuterated α-T, i.e., ∅ = $\Sigma(d_x$-α-T)/(d_0-α-T), which is d_3-*RRR*-α-T/d_0-*RRR*-α-T in experiment 1 and (d_6-*RRR*-α-T + d_3-*SRR*-α-T)/d_0-*RRR*-α-T in experiment 2. The complete set of data are presented in Table 3. (Note that the data presented for plasma and RBC are average values.) A comparison of the results from animals that were perfused with saline with those that were not (day 4 in both experiments) indicates that the results were not affected by the presence of blood in the tissue.

In both experiments, plots of ∅ vs time were linear for almost all tissues. This result was not anticipated and seems remarkable considering the fact that each point in the plots was obtained from a different animal. Some examples of these plots are shown in Figure 1.

In view of the demonstrated linearity of ∅ with time, the data were fitted by the method of least squares to the equation ∅ = kt, where t is time and k is a proportionality constant, the value of which depends on the tissue, diet, etc. The results are shown in Table 4.

The two k values obtained for each tissue in experiment 2 (i.e., with or without inclusion of the final point for day 154) are in satisfactory agreement except in the case of lung. If this tissue is excluded from consideration, with only one or two exceptions the k values derived from experiment 2 are somewhat lower than the k values derived from experiment 1. This means that the rate of uptake of α-T by the rats is smaller when the vitamin is provided as the mixture of two stereoisomers in experiment 2 than when it is provided as the natural stereoisomer in experiment 1.

The reciprocal of each k value listed for experiment 1 in Table 4 yields the apparent half-life, $t_{1/2}$, in days for natural *RRR*-α-T in each tissue; $t_{1/2}$ is the time it takes for the net amount of ingested deuterated *RRR*-α-T taken up by a tissue to equal the amount of the nondeuterated *RRR*-α-T remaining. This time is shortest for the lung ($t_{1/2} \approx 7$ days, experiment 1), liver and small intestine and is longest for the brain, testes ($t_{1/2} \approx 30$ days, experiment 1) and spinal cord ($t_{1/2} \approx 76$ days, experiment 1).

Figure 2 shows the time dependence of the discrimination in the net uptake of d_6-*RRR*-α-T vs d_3-*SRR*-α-T in plasma, RBC, liver and brain of rats fed the diet containing equal amounts of the acetates of these diastereoisomers (experiment 2). After a single day there is a 1.4-fold enrichment of d_6-*RRR*-α-T in the plasma, the enrichment increasing to ca. 2.5 over the 5-mo duration of this experiment. The RBC consistently show a higher enrichment in d_6-*RRR*-α-T than the plasma throughout the experiment; the enrichment ratio, i.e., $(RRR/SRR)_{rbc}/(RRR/SRR)_{plasma}$ has a mean value of 1.35 ± 0.13 (standard deviation for 30 values).

Three remarkable results are also shown in Figure 2. First, the liver, in contrast to all the other tissues, shows an initial excess, by a factor of two, of d_3-*SRR*-α-T. This lasts for ca. 3 wk until eventually the liver begins to show a slight excess of d_6-*RRR*-α-T. The initial excess of the *SRR*-epimer is similar to the result reported from an early experiment by Weber et al. (12), in which fasted rats received single doses of either 2*S*,4′*RS*,8′*RS*-α-T or 2*R*,4′*RS*,8′*RS*-α-T radioactively labeled acetates. In that experiment, the average level of the 2*S*-diastereoisomers found in the liver half an hour after the doses were orally administered was approximately twice that of their 2*R*-epimers (12). However, after two hours the situation was reversed and the 2*R*-diastereoisomers exceeded their 2*S* counterparts by a small margin. Although in our own experiment the rats were deprived of food for at least four hours before their livers were removed, it is very likely that some food was still present in the gut at the time they were killed. There will, therefore, have been a continuing infusion of vitamin E into the animals. This may explain why our liver data appear to be similar to the trends noted in the very early stages of the single dose experiment (12).

Second, the brain shows an extraordinary, progressive discrimination in favor of natural *RRR*-α-T, especially after one month, with the *RRR/SRR* ratio exceeding a value of five after five months and showing no sign of leveling off. This result has been confirmed in subsequent related experiments. In one experiment, two rats were placed on the diet used in experiment 2 (i.e., 1:1 d_6-*RRR*-α-T/d_3-*SRR*-α-T acetates) immediately after weaning and were found to have in their brains d_6-*RRR*-α-T/d_3-*SRR*-α-T ratios of 5.7 and 4.8 after 120 and 128 days, respectively. In a second experiment in which two weanling rats

TABLE 3

Time-Dependence of ∅ (Ratio of [Total] Deuterated α-T to Nondeuterated α-T) for Blood and Tissues in Experiments 1 and 2[a]

		Day											
Tissue	Expt.	1	2	4	4	8	16	31	32	64	65	65	154
Lung	1	0.08	0.17	0.53	0.40	0.80	1.80	3.30			10.20	6.90	
	2	0.08	0.14	0.40	0.31	0.86	1.60		2.00	2.80			25.00
Liver	1	0.16	0.35	0.79	0.65	1.00	1.90	3.40			6.30	7.20	
	2	0.32	0.47	0.96	0.90	1.70	2.30		3.10	5.60			17.00
Small intestine	1	0.06	0.37	1.30	0.51	0.78	1.90	3.70			6.30	6.40	
	2	0.08	0.19	0.43	0.42	0.94	1.80	2.90	4.50				11.00
Plasma[b]	1	0.50	0.50	0.77		1.00	2.00	3.40			6.10		
	2	0.15	0.33	0.65		1.00	1.70		2.50	3.80			12.00
Kidney	1	0.05	0.09	0.27	0.25	0.49	1.30	2.70			5.60	5.80	
	2	0.04	0.08	0.20	0.22	0.73	1.20		2.10	3.20			9.30
Red blood cells[b]	1	0.15	0.35	0.73		1.30	1.90	3.20			5.20		
	2	0.14	0.26	0.65		1.10	1.70		2.40	3.70			9.60
Heart	1	0.02	0.13	0.13	0.11	0.28	0.88	2.30			4.70	4.80	
	2	0.01	0.03	0.10	0.13	0.28	0.81		1.10	3.10			12.00
Thymus	1	0.03	0.07	0.30	0.36	0.55	1.29	1.62			4.50	4.67	
	2	0.03	0.06	0.31	0.31	0.75	1.39		2.18	3.93			8.46
Muscle (biceps femoris)	1	0.02	0.04	0.11	0.09	0.20	0.67	1.70			4.70	3.80	
	2	0.02	0.03	0.08	0.09	0.22	0.56		1.20	3.50			9.70
Muscle (tibialis anterior)	1	0.01	0.03	0.09	0.10	0.21	0.62	1.33			4.89	3.61	
	2	0.01	0.02	0.06	0.06	0.19	0.54		1.42	2.66			12.35
Muscle (soleus)	1	0.02	0.04	0.10	0.12	0.26	0.68	1.47			3.92	4.19	
	2	0.02	0.04	0.09	0.10	0.25	0.64		1.21	2.99			10.12
Spleen	1	0.13	0.48	0.74	0.66	0.98	2.00	3.20			6.40	1.80	
	2	0.11	0.21	0.53	0.53	0.99	1.20		2.20	4.10			11.00
Muscle (tensor fascia latae)	1	0.02	0.03	0.10	0.10	0.24	0.74	1.58			2.98	4.50	
	2	0.02	0.04	0.09	0.10	0.24	0.60		1.21	3.44			9.84
White adipose tissue (retroperitoneal)	1	0.03	0.03	0.04	0.08	0.21	0.46	1.30			3.40	3.20	
	2	0.02	0.02	0.10	0.12	0.26	0.52		ND[c]	3.50			5.50
Aorta	1	0.02	0.07	0.10	0.10	0.28	0.64	1.31			2.83	2.95	
	2	0.03	0.05	0.11	0.10	0.22	0.56		0.95	2.25			5.40
Brown adipose tissue (interscapular)	1	0.01	0.02	0.07	0.08	0.14	0.43	1.17			2.56	3.06	
	2	0.01	0.01	0.06	0.07	0.17	0.46		0.98	2.46			8.71
Diaphragm	1	0.02	0.03	0.09	0.12	0.22	0.59	1.41			2.00	3.51	
	2	0.02	0.02	0.09	0.09	0.21	0.57		1.17	2.72			8.49
Skin	1	0.02	0.04	0.12	0.10	0.25	0.79	1.37			2.72	2.75	
	2	0.02	0.04	0.11	0.11	0.27	0.65		1.29	2.30			6.98
White adipose tissue (inguinal)	1	0.01	0.02	0.07	0.08	0.17	0.45	0.98			2.26	2.26	
	2	0.01	0.02	0.07	0.08	0.19	0.53		0.94	2.12			5.95
Brain	1	<0.01	0.02	0.06	0.03	0.06	0.21	0.64			1.50	2.80	
	2	<0.01	0.01	0.04	0.03	0.10	0.25		0.51	1.20			4.10
Testes	1	0.01	0.04	0.14	0.12	0.21	0.31	0.79			1.90	2.00	
	2	0.02	0.03	0.08	0.08	0.20	0.56		0.61	1.70			7.10
White adipose tissue (epididymal)	1	<0.01	0.01	0.03	0.07	0.16	0.32	0.84			1.70	1.94	
	2	<0.01	0.02	0.01	0.01	0.17	0.36		0.70	1.56			4.02
Spinal cord	1	0.01	0.02	0.04	0.04	0.07	0.15	0.39			0.90	0.78	
	2	0.01	0.01	0.04	0.05	0.10	0.15		0.28	0.54			1.56

[a]Ratios of the deuterated to nondeuterated forms, d_3-*RRR*-α-T/d_0-*RRR*-α-T (experiment 1) and (d_6-*RRR*-α-T + d_3-*SRR*-α-T)/d_0-*RRR*-α-T (experiment 2), respectively, are given for various times after switching the rats to the diets containing the deuterated α-tocopherols. In experiment 1, only the animal corresponding to the data for the first set of day 4 results was perfused with saline. In experiment 2, all animals were perfused except the animal corresponding to the second set of day 4 results.

[b]Mean values for blood drawn from several rats (except day 154): number of rats (day no.)—4 (1), 7 (2), 6 (4), 4 (8), 3 (16), 2 (31), 2 (32), 2 (64), 2 (65). Single values for day 111 (experiment 2 only) were 5.94 and 10.35 for plasma and red blood cells, respectively.

[c]ND, not determined.

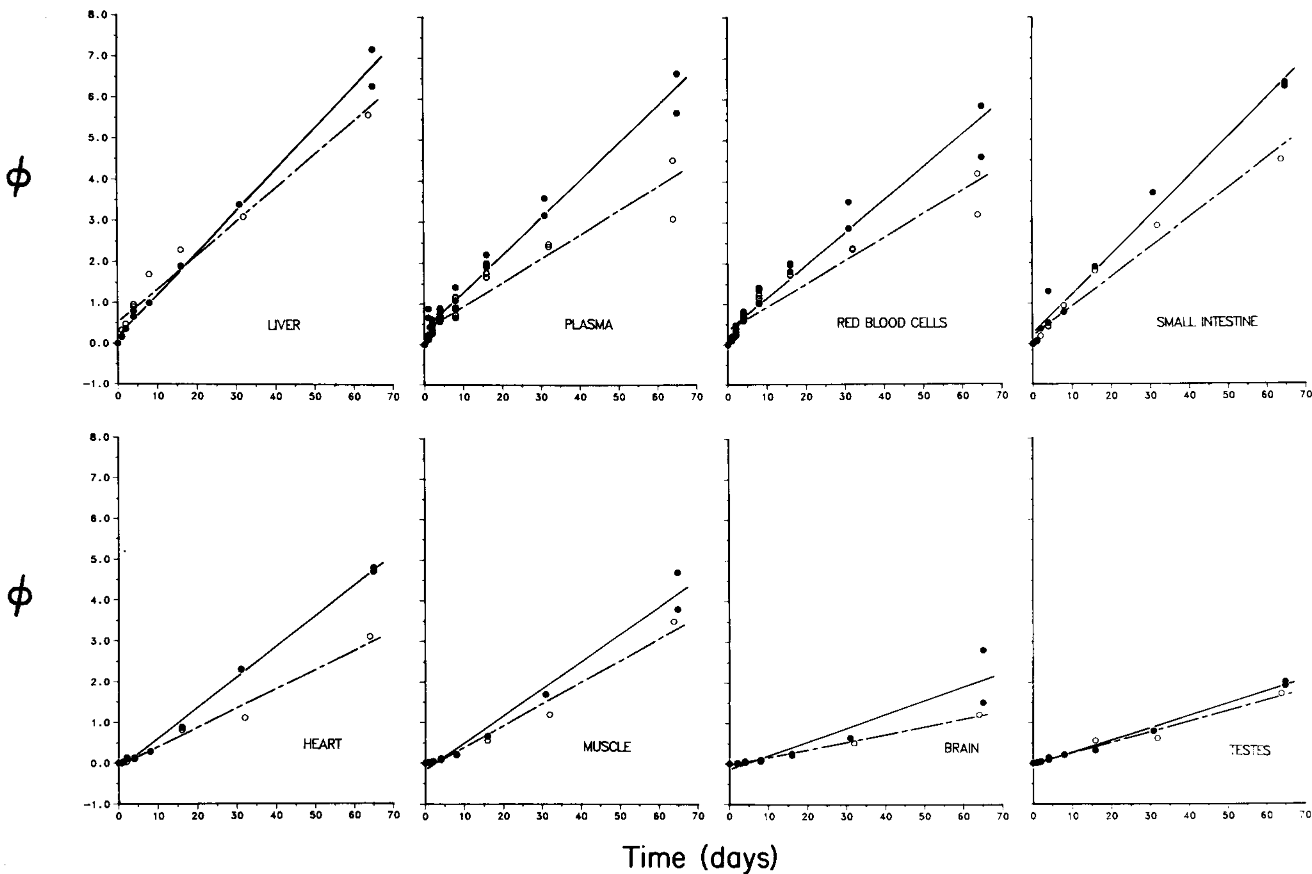

FIG. 1. Plots of ∅ [ratio of the total amount of deuterated to nondeuterated α-tocopherols, $\Sigma(d_x\text{-}\alpha\text{-T})/(d_0\text{-}\alpha\text{-T})$] vs time for liver, plasma, red blood cells, small intestine, heart, muscle (soleus), brain and testes. Each graph includes data points from experiment 1 (d_3-*RRR*-α-T/d_0-*RRR*-α-T; solid line) and experiment 2 [(d_6-*RRR*-α-T + d_3-*SRR*-α-T)/d_0-*RRR*-α-T; broken line)]. Lines were obtained by a least squares fit of the data collected over a 64–65 day period for each experiment. The data ponts are from single rats (9 and 8 points for each tissue in experiments 1 and 2, respectively, and 30 data points for plasma and red blood cells in both experiments).

TABLE 4

Values of k and Apparent Half-Lives, $t_{1/2}$, of α-T in Tissues and Blood[a]

	Experiment 1	Experiment 2		
Tissue	k (Day 65)[b]	k (Day 64)[b,c]	k (Day 154)[b]	$t_{1/2}$[d]
Lung	0.132 ± 0.011	0.045 ± 0.006	0.151 ± 0.017	7.6
Liver	0.102 ± 0.003	0.082 ± 0.006	0.105 ± 0.004	9.8
Small intestine	0.096 ± 0.004	0.072 ± 0.005	0.070 ± 0.002	10.4
Plasma	0.091 ± 0.003	0.059 ± 0.004	0.067 ± 0.003	10.9
Kidney	0.089 ± 0.001	0.052 ± 0.004	0.059 ± 0.002	11.2
Red blood cells	0.080 ± 0.004	0.057 ± 0.004	0.068 ± 0.003	12.5
Heart	0.075 ± 0.002	0.047 ± 0.003	0.076 ± 0.005	13.3
Thymus	0.070 ± 0.003	0.062 ± 0.003	0.055 ± 0.002	14.4
Muscle (biceps femoris)	0.067 ± 0.004	0.053 ± 0.003	0.063 ± 0.002	14.9
Muscle (tibialis anterior)	0.066 ± 0.005	0.043 ± 0.001	0.078 ± 0.006	15.1
Muscle (soleus)	0.063 ± 0.002	0.046 ± 0.002	0.065 ± 0.003	15.8
Spleen	0.060 ± 0.016	0.062 ± 0.003	0.070 ± 0.002	16.6
Muscle (tensor fascia latae)	0.059 ± 0.005	0.053 ± 0.003	0.064 ± 0.002	17.0
White adipose tissue (retroperitoneal)	0.052 ± 0.002	0.056 ± 0.002	0.038 ± 0.003	19.3
Aorta	0.045 ± 0.001	0.035 ± 0.001	0.035 ± 0.000	22.2
Brown adipose tissue (interscapular)	0.044 ± 0.002	0.038 ± 0.002	0.056 ± 0.003	22.6
Diaphragm	0.043 ± 0.005	0.043 ± 0.001	0.054 ± 0.002	23.0
Skin	0.043 ± 0.001	0.037 ± 0.001	0.045 ± 0.001	23.4
White adipose tissue (inguinal)	0.035 ± 0.001	0.033 ± 0.001	0.038 ± 0.001	28.2
Brain	0.034 ± 0.005	0.019 ± 0.001	0.026 ± 0.001	29.4
Testes	0.030 ± 0.001	0.026 ± 0.002	0.044 ± 0.003	33.3
White adipose tissue (epididymal)	0.029 ± 0.001	0.025 ± 0.001	0.026 ± 0.000	34.8
Spinal cord	0.013 ± 0.001	0.008 ± 0.000	0.010 ± 0.000	76.3

[a]Obtained by least squares analysis of data fit to the equation ∅ = kt, where ∅ is the ratio of (total) deuterated to nondeuterated α-tocopherols and t is time in days. Units are day^{-1} (± standard deviation) for k and days for $t_{1/2}$.

[b]Duration of experiment.

[c]Results obtained by excluding the one additional data point for day 154.

[d]Calculated from the reciprocal of the k values from experiment 1.

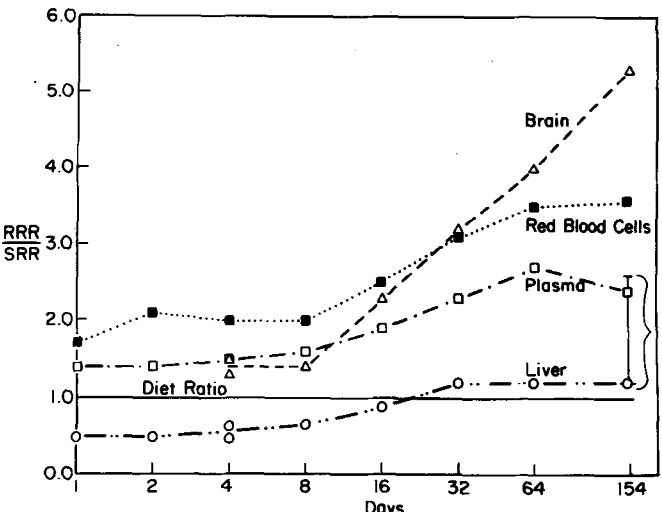

FIG. 2. Time-dependence of the discrimination in the net uptake of deuterated *RRR*- and *SRR*-α-tocopherols (d_6-*RRR*-α-T/d_3-*SRR*-α-T) into plasma, red blood cells, liver and brain of male rats fed a diet containing equal amounts of the corresponding tocopherol acetates (experiment 2). Points for plasma and red blood cells represent mean values from two or more animals (except for day 154). The points for liver and brain are from single animals. The bar at 154 days represents the full range in the *RRR/SRR* ratio found at 154 days for all of the other tissues examined (see Table 4).

were placed on a diet with a d_6-*RRR*-α-T/d_3-*SRR*-α-T acetate ratio of 0.5, the d_6-*RRR*-α-T/d_3-*SRR*-α-T ratios in the rats' brains were 2.2 and 2.5 after 64 days; that is, relative to the dietary ratio, the enrichments of d_6-*RRR*-α-T to d_3-*SRR*-α-T were 4.4 and 5.0, respectively.

Third, all the other tissues examined (except brain, RBC, liver and spinal cord; see Table 5 for complete data) show discriminations that after one month lie between that of plasma on one hand and liver on the other (see bar at day 154 in Fig. 1).

Analysis of fecal material from the large intestine (Table 6) reveals that the hydrolysis of the dietary tocopherol acetates in the gut was incomplete, particularly in experiment 2. It is also very clear that *RRR*-α-T-Ac underwent hydrolysis to a greater extent than *SRR*-α-T-Ac. This latter observation is probably the reason why the amount of fecal d_6-*RRR*-α-T was generally larger than the amount of d_3-*SRR*-α-T, and the ratio of α-T-Ac to α-T was generally higher in experiment 2 than in experiment 1.

The data in Table 6 also show that the original, non-deuterated tocopherol persists in the gut longer than would be expected after switching the animals to a diet containing 100% deuterated tocopherol. From the GC-MS data provided in Table 1, the apparent limiting ratio of d_3-*RRR*-α-T to d_0-*RRR*-α-T in experiment 1 can be calculated to be 100/1.3 = 76.9. Similarly, the apparent limiting ratio in experiment 2 (i.e., for a 1:1 mixture of

TABLE 5

The Dependence with Time of the Discrimination in the Uptake of Deuterated *RRR*- and *SRR*-α-Tocopherols in Experiment 2[a]

	Day								
Tissue	1	2	4	4	8	16	32	64	154
Lung	1.40	1.30	1.30	1.40	1.20	1.50	1.90	1.90	2.60
Liver	0.49	0.49	0.47	0.62	0.67	0.89	1.20	1.20	1.20
Small intestine	1.30	1.30	1.20	1.20	1.10	1.40	1.50	1.70	1.80
Plasma[b]	1.40	1.40	1.50		1.60	1.90	2.30	2.70	2.40
Kidney	1.60	1.50	1.30	1.40	1.30	1.50	1.80	1.20	1.80
Red blood cells[b]	1.70	2.10	2.00		2.00	2.50	3.10	3.50	3.60
Heart	0.96	0.99	1.00	1.40	0.88	1.20	0.99	1.40	1.90
Thymus	1.60	1.44	1.32	1.40	1.25	1.61	1.81	1.84	1.58
Muscle (biceps femoris)	1.40	1.40	1.30	1.40	1.30	1.40	1.70	1.90	1.70
Muscle (tibialis anterior)	—[c]	—[c]	1.33	1.39	1.37	1.74	2.22	1.99	2.12
Muscle (soleus)	—[c]	1.49	1.38	1.47	1.31	1.64	1.92	2.12	2.22
Spleen	1.40	1.40	1.10	1.20	0.97	1.50	1.80	1.30	2.10
Muscle (tensor fascia latae)	—[c]	1.51	1.26	1.42	1.23	1.53	1.69	2.02	1.65
White adipose tissue (retroperitoneal)	1.40	1.30	1.40	1.40	1.20	1.50	—[d]	2.10	1.90
Aorta	1.54	1.25	1.16	1.34	1.20	1.41	1.73	1.70	1.68
Brown adipose tissue (interscapular)	—[c]	—[c]	1.66	1.61	1.42	1.55	1.57	1.40	1.19
Diaphragm	—[c]	1.04	1.40	1.40	1.36	1.66	1.92	2.07	2.22
Skin	1.04	1.00	1.14	1.22	1.09	1.33	1.26	1.44	1.39
White adipose tissue (inguinal)	—[c]	1.65	1.56	1.52	1.28	1.52	1.60	1.61	1.55
Brain	—[c]	—[c]	1.50	1.30	1.40	2.30	3.20	4.00	5.30
Testes	1.60	1.40	1.40	1.40	1.30	1.70	1.80	1.90	2.00
White adipose tissue (epididymal)	—[c]	—[c]	—[c]	—[c]	1.33	1.99	1.89	2.25	2.14
Spinal cord	—[c]	—[c]	1.34	1.31	1.24	1.70	2.40	2.89	3.69

[a]Values of the ratio d_6-*RRR*-α-T/d_3-*SRR*-α-T are given for various times after switching the rats to the diet containing equal amounts of the two deuterated α-tocopherols.

[b]Mean values for blood drawn from several rats (except day 154): number of rats (day no.)—4 (1), 7 (2), 6 (4), 4 (8), 3 (16), 2 (32), 2 (64). Single values for day 111 were 2.66 and 4.17 for plasma and red blood cells, respectively.

[c]Insufficient deuterated tocopherols available to determine ratio accurately.

[d]Not determined.

TABLE 6

Absolute Concentrations of α-T and α-T Acetate (per Wet Wt) and Ratios d_3-*RRR*-α-T/d_0-*RRR*-α-T (Experiment 1), d_6-*RRR*-α-T/d_3-*SRR*-α-T and (d_6-*RRR*-α-T + d_3-*SRR*-α-T)/d_0-*RRR*-α-T (Experiment 2) in Fecal Material Recovered from Large Intestine

Experiment 1				Experiment 2					
		α-Tocopherol			α-Tocopherol acetate		α-Tocopherol		
Day	α-T-Ac (nmol/g)	nmol/g	d_3-*RRR*/d_0-*RRR*	Day	nmol/g	d_6-*RRR*/d_3-*SRR*	nmol/g	d_6-*RRR*/d_3-*SRR*	(d_6-*RRR* + d_3-*SRR*)/d_0-*RRR*
1	10	87	3.66	1	36	0.22	99	1.34	4.23
2	8	71	10.55	2	19	0.43	99	0.97	6.53
4	18	95	20.98	4	7	0.74	76	0.95	8.76
4	19	75	17.04	4	42	0.45	109	1.06	12.64
8	17	83	20.06	8	33	0.26	95	1.08	25.41
16	16	95	24.91	16	90	0.21	108	1.95	31.90
31	41	67	36.08	32	33	0.29	99	1.20	12.25
65	34	84	69.91	64	137	0.33	66	2.59	58.74
65	8	78	33.20	154	36	0.42	51	1.13	44.48

d_3-*SRR*-α-T and d_6-*RRR*-α-T) can be calculated to be (100 + 100)/(0.26 + 0.13) = 513. It is evident that these limits are not attained in either experiment.

DISCUSSION

The advantage of using deuterium-labeled α-T in conjunction with GC-MS for studies of relatively long-term uptake and discrimination of vitamin E is evident from the present results. Our two experiments have yielded remarkably consistent results for the net rates of uptake of deuterated α-T by various tissues. The plots of Ø vs time for the tissues and blood show an unexpected and surprising degree of linearity. Even though there is a steady influx of vitamin E from the diet, this fact alone is not sufficient to explain the linearity. Nevertheless, the empirically observed linearity of Ø has allowed us to obtain the first comprehensive picture of the biokinetics in the tissues and blood of α-T acquired under normal dietary conditions. Particularly noteworthy is the large range in the rates of net uptake of deuterated α-T into different tissues. This is dramatically illustrated by the α-T half-lives ($t_{1/2}$), which range from 8 days in the lung to 76 days in the spinal cord (see Table 4). Of course, the animals underwent significant growth during the experiment (see Table 2) and therefore the total quantity of tocopherol was increasing in many organs and tissues because the size of the organs and tissues was increasing. Growing tissue would be expected to take up more tocopherol than it loses, and the new cells would be expected to contain mainly the deuterated (i.e., new) α-T. Growth would therefore have the effect of increasing the Ø values compared to the values that might be obtained in mature animals of stable weight. In growing tissues, the $t_{1/2}$ values will therefore tend to underestimate somewhat the actual time for a 50% turnover of α-T.

It is interesting to note that the Ø values were higher in lung, liver and small intestine than in plasma. This is easy to understand for the small intestine, since it is here that the new (deuterated) α-T is being absorbed from food containing only deuterated α-T. We suggest that the Ø values for the other two organs are higher than in plasma because uptake of α-T occurs most rapidly soon after feeding, when both the total α-T concentration and the Ø ratio in the plasma are temporarily elevated (25). This enhanced rate of absorption at elevated α-T levels in plasma may be combined with a fairly rapid cell turnover in these tissues (i.e., the death of old cells with "old" d_0-*RRR*-α-T and the birth of new cells with "new" d_x-α-T).

For organs whose weights remained essentially the same or increased only marginally during the experiment (e.g., brain, testes and heart), $t_{1/2}$ must correspond quite closely to the time it took for 50% of the natural α-T to be lost from a tissue by all chemical and physical processes. It remains to be seen to what extent the rate of turnover is determined by chemical consumption (by, for example, oxidation of α-T by lipid peroxyl radicals, which is not repaired by ascorbate or other water-soluble reducing agents [2; 26 and references cited therein]) and by physical loss (e.g., cell death, exchange into plasma or lymph).

A second major aspect of this work is that it provides direct information regarding the pattern of tissue discrimination between two of the eight diastereoisomers of α-T. This gives us new insights into the reasons for the different bioactivities of individual stereoisomers, including, for example, the difference between natural and *all-rac*-α-T.

The results obtained from fecal material suggest that discrimination in favor of the natural compound begins with the hydrolysis of the α-T acetates in the gut, i.e., before there has even been any absorption of the free phenol into the lymph. The simplest explanation for this would be that *RRR*-α-T-Ac is more rapidly hydrolyzed than *SRR*-α-T-Ac, presumably because of the chirality of the active enzymes in the pancreatic juice (e.g., carboxyl ester hydrolase) or the bile salts that are necessary for hydrolysis to occur (25,27) or to both. However, this explanation is not consistent with the result obtained by Weber et al. (12), which showed that the 2*S*-diastereoisomers are absorbed more rapidly within the first half-hour after dosing. A possible explanation that reconciles the results from both experiments is that the hydrolysis of

the 2*S*-diastereoisomers is faster but is more susceptible to inhibition by free tocopherol. The effects of inhibition and chiral discrimination upon the in vitro enzymic hydrolysis of α-T acetates are currently being investigated.

Our discovery that a small percentage of the α-T present in the fecal material is undeuterated after two months (experiments 1 and 2) and even after five months (experiment 2; see Table 5) suggests that α-T is "returned" to the gut from at least some tissues in the body of the rat. We presume that this return occurs by transport in the plasma and lymph with eventual excretion in the bile (experiments underway).

RBC exhibit a moderate chiral selectivity, relative to plasma, in favor of *RRR*-α-T [$(RRR)/(SRR)_{rbc}/(RRR)/(SRR)_{plasma}$ = 1.35, see Fig. 2]. We presume that chiral discrimination by the (chiral) phospholipids, cholesterol, proteins, etc., will be accentuated by the more structured lipid environment of the RBC membranes compared with the more "relaxed" environment of the plasma lipoproteins. In this regard, attempts to measure chiral effects in model phospholipid vesicles by various nuclear magnetic resonance methods have not been successful (28).

The unique behavior of the liver, in which there is an initial discrimination in favor of the unnatural *SRR*-α-T for ca. 3 wk, suggests that it possesses a mechanism by which *SRR*-α-T is selectively extracted from the lymph (25) and/or a mechanism that selectively "exports" *RRR*-α-T into lipoproteins that enter the blood plasma. Lipid "export" from the liver is known to involve the very low density lipoproteins (VLDL) and is likely to be accompanied by vitamin E "export." Indeed, an α-T transport protein, which is present in rat liver cytosol but not in other tissues (29–32), has been identified and shown to be fairly specific for the natural stereoisomer (31). We therefore suggest that this protein is active in transporting *RRR*-α-T into the lipoproteins synthesized in the liver, i.e., *RRR*-α-T is selectively "exported." There is, presumably, also some mechanism for the removal of *SRR*-α-T from the liver. However, it appears to take some weeks to become fully activated.

The very strong long-term discrimination by rat brain in favor of (natural) *RRR*-α-T and the very slow uptake of α-T into spinal cord have potential consequences for human nutrition and the treatment of neurological diseases associated with vitamin E deficiency (33,34). The high degree of discrimination implies that the rat brain will take up less of the *ambo*-α-T than *RRR*-α-T from diets that contain the same amount of both. The biokinetic data support this. The difference between the k values for experiments 1 and 2 is most marked for the brain (see Table 4). This result strongly suggests that a diet (dose) of natural α-T will be significantly more "available" to the brain than an equivalent diet (dose) of synthetic *all-rac*-α-T.

It seems likely that the high discrimination shown by the brain and to a lesser extent by the spinal cord is a result of the "blood-brain barrier." Possibly, α-T enters these tissues by passive diffusion across several membranes. This would be slow, and by analogy to the RBC, would tend to favor the natural stereoisomer.

The other tissues show no enhancement of the *RRR/SRR* ratio relative to plasma. This is probably because α-T uptake by tissue occurs in conjunction with the uptake of other lipidic material, there being, therefore, little chance of chiral discrimination at the point of entry of the tocopherol into the cell. Thus, α-T is known to be present in all lipoprotein fractions, and two mechanisms have been identified by which α-T is transferred from human lipoproteins to tissue. First, the specific, high affinity, low density lipoprotein (LDL) receptor mechanism (35) has been shown to be involved in the delivery of vitamin E from human LDL to human fibroblast cells (36,37). Second, α-T present in human chylomicrons has been shown to transfer to human erythrocytes and fibroblasts during the hydrolysis of triglycerides by lipoprotein lipase, and for this transfer to occur the lipase must itself bind to the cell membrane (38). The second of these two mechanisms is probably the more important in the rat (39); the *RRR/SRR* ratio in most tissue should at least parallel the *RRR/SRR* ratio in the plasma. The mechanism by which α-T is transferred to or from tissue by the high density lipoproteins (HDL) has not apparently been identified, although α-T in human HDL has been shown to transfer readily to human erythrocytes (40).

Finally, our results raise serious questions regarding the validity of and reliance upon current animal bioassays for evaluating the biopotencies of different tocopherols. As far as the stereoisomers of α-T are concerned, it would seem that both the duration of the test period and the tissue(s) that give the observed symptoms of deficiency and cure could affect the derived biopotency.

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Studies of Lipoproteins and Fatty Acids in Maternal and Cord Blood of Two Racial Groups in Trinidad

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The high mortality rate from coronary heart disease (CHD) among Indians compared to Negroes in Trinidad led us to test plasma lipid profiles to see whether dietary or genetic factors might be involved. There were no interracial differences in the composition of plasma cholesterol ester fatty acids of the tested women and neonates. This finding suggests that dietary fat does not account for the interracial difference in CHD, nor does the cause appear to be due to genetic differences in lipid profiles, as there was no significant difference between values for plasma triglycerides, total cholesterol, high density lipoprotein (HDL) cholesterol, apo-I, apo-II, apo B or cholesterol ester fatty acids in the cord blood of each racial group.

Blood samples were collected from 69 nonpregnant and 71 postpartum, fasted Negro and Indian women. Also taken were 71 umbilical cord blood samples. The mean triglyceride level was significantly lower in the Negro nonpregnant and postpartum women than in the Indians. HDL cholesterol and apo-I values were lower in the Indian women. There were no significant differences in the total cholesterol and apo B measurements. The triglyceride values for postpartum women were higher than those of the nonpregnant Negroes and Indians (75% and 47%, respectively), whereas the total cholesterol and HDL cholesterol, apo A-I and apo A-II ranged from 9% to 29% higher in the postpartum women. Apo B was about 40% higher postpartum in both ethnic groups.

The high CHD rate of Indians in Trinidad cannot be explained by dietary factors, plasma total cholesterol or fatty acid composition. However, the lower level of HDL cholesterol and plasma A-I could play a role in the higher CHD rate in Indians.
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Coronary heart disease (CHD) risk increases with increasing concentration of total or low density lipoprotein (LDL) cholesterol and decreases with an increased high density lipoprotein (HDL) cholesterol concentration (1,2). Elevated plasma triglycerides and very low density lipoprotein (VLDL) levels have also been associated with an increased CHD risk, but this association may be related to the effect of other lipoproteins, such as the reduction of HDL (3–5). However, a reduced level of HDL cholesterol is found in some populations where the incidence of ischemic heart disease is rare (6,7). It has been suggested that the prediction for CHD risk could be improved by simultaneous determination of lipoproteins and apoproteins (8).

CHD is the highest single cause of mortality in Trinidad (9). The difference between the rates of CHD for the Negro and Indian populations has been attributed to the high prevalence of diabetes mellitus and to the elevated concentrations of serum cholesterol evident in Indians in Trinidad and elsewhere (10,11). A 1982 study by Miller et al. (12) confirmed that Indian men in Trinidad have an unfavorable lipoprotein profile, with an elevation of VLDL and LDL triglycerides and cholesterol and a reduction in HDL cholesterol. But Miller's 1985 study (13) on serum HDL subclasses, testosterone and sex-hormone-binding globulin did not offer any explanation for a lower serum HDL level in Indians compared with Negroes in Trinidad.

Dietary and genetic factors may explain the differences in mortality rate from CHD between these two racial groups. The quantity and composition of dietary fat affect serum lipids and lipoproteins. One usually associates a high serum cholesterol level with populations consuming high dietary fat (14). However, the only food survey (15) carried out in Trinidad showed that Indians purchase more cereals, grains, vegetables and fish and less milk, dairy products, oil and fat than other racial groups in Trinidad. To date, these data have not been substantiated with detailed studies of dietary consumption. Analysis of the fatty acid composition of plasma cholesterol esters provides a limited assessment of both the composition (16) and quantity (17) of consumed dietary fat. Reduced concentration of dienoic fatty acid in plasma cholesterol esters has also been associated with development of myocardial infarction (18).

This study investigates the differences in plasma lipids, lipoproteins, apoproteins and fatty acid composition of plasma cholesterol ester in normal women of Negro and Indian descent in Trinidad. The comparative study was extended to include plasma from cord blood and from women after delivery, to see if there were differences in lipid profile at birth. While marked differences in the fatty acid composition of cholesterol esters of maternal and cord blood samples have been found within races (19), the difference between races has not been studied.

SUBJECTS AND METHODS

The postpartum subjects for this study were all women who came for delivery during the month of April 1984 at the Sangre Grande County General Hospital, Trinidad. This hospital serves the entire St. Andrews area, where the major ethnic groups are Negroes and Indians. The ethnicity of each participant was defined by the race of the subjects' grandparents ("Indian" or "Negro" if three of four grandparents were purely of that race). The study

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had the permission of the Government Chief Medical Officer, and participants gave informed consent to all procedures.

Participants were free of gestational complications, and delivery occurred between 37 and 40 wk. Patients fasted from 10 to 13 hr prior to delivery, and blood was collected by antecubital venipuncture within 15 min postpartum. Cord blood was drained from the placental end of the umbilical cord immediately after transection. The blood samples were anticoagulated with EDTA (1 mg/ml) and kept on ice until plasma could be separated by a 15-min centrifugation at 1500 × g. This was accomplished within an hour of collection, after which samples were kept frozen at −20 C and shipped to participating laboratories.

Blood was also obtained from fasted nonpregnant women who were attending the Family Planning Clinic at Sangre Grande, after the purpose of the study had been explained to them. They were not on any contraceptive pill at the time of the study.

All subjects were interviewed, and details of age, occupation of spouse, dietary habits, sources and amounts of dietary fat, alcohol use, tobacco use, reproductive history and gestational history were recorded on a standard questionnaire. Physical parameters recorded included adult height and weight (antepartum of mothers) and neonatal weight, sex, length and head circumference.

Determination of total HDL cholesterol and triglycerides. The procedure used was that described in the Lipid Research Clinics Program Manual (20). For HDL cholesterol determination, 0.15 ml of 1 M $MnCl_2$ and 0.12 ml of 35 mg/ml heparin (Riker Laboratories, Northbridge, California) were added to a 3-ml aliquot of plasma.

Cholesterol of the supernatant fraction and the plasma triglyceride was analyzed with a Technicon Auto Analyzer II (Technicon Instruments Corp., Terrytown, New York) (20). For cholesterol analysis, the coefficient of variation was 2%. For triglyceride analysis, they were 3% and 4%, respectively.

Determination of apo A-I and A-II. The method used was as described by Albers et al. (21) and Cheung and Albers (22). Plasma aliquots were diluted with an equal volume of 8.4 M, 1,1,3,3-tetramethylurea (TMU) and mixed. The TMU plasma mixture was diluted with Tris-Urea and incubated at room temperature. Agarose radial immunodiffusion plates were prepared as described (23). From each sample, 0.4 μl was drawn and added to duplicate wells chosen in different quadrants of the plates. The plates were placed in a humid chamber at 37 C until the precipitate rings attained their final size, which was usually after 48 hr. The precipitate rings were measured and, using the square of the diameter of the precipitate ring and the concentration of the standard, the values for apo A-I and apo A-II for each sample were computed. The coefficient of variation for the assay was 7% for apo A-I and 6–10% for apo-II.

Determination of apo B. Apo B was quantified by the double antibody immunoassay technique described by Albers et al. (24). Briefly, 10 μl of plasma was diluted with 90 μl of RIA buffer (20 mM borate, 150 mM NaCl, 1 mM disodium EDTA, 6% bovine serum albumin, pH 8.0) followed, in order, by addition of 100 μl of ^{125}I-LDL (65 μg/ml) and 100 μl of anti-LDL (about 1:240 dilution). The mixture was incubated for 24 hr at 4 C. Then, 100 μl of diluted (about 1:50) normal rabbit serum and 300 μl of sheep anti-rabbit IgG were added to each tube, mixed, and incubated for 14–18 hr at 4 C. The samples were centrifuged, the supernatant was decanted and the precipitate was washed twice with RIA buffer. A set of standards and quality control samples were also included in the assay. Radioactivity in all tubes was determined. The percentage ^{125}I-LDL bound was calculated, and the relationship between antigen bound and log dose was linearized by unweighted least square regression of the logit of the percentage antigen bound vs log dose.

Fatty acid determination. The fatty acid composition of five samples of dietary oil, collected from the city of Sangre Grande, as well as cholesterol esters from 33 pairs of maternal and cord sera were determined. Lipids were extracted by the method of Folch et al. (25) and separated by thin layer chromatography on 0.5-mm silica gel H plates (E. Merck, Darmstadt, Federal Republic of Germany); the solvent system comprised petroleum ether (B.P. 40–60 C), diethyl ether and glacial acetic acid (160:30:5, v/v/v). Lipid fractions were localized with rhodamine G, scraped into extraction tubes and extracted into chloroform. Cholesterol esters were hydrolyzed, and the fatty acids were methylated with BF_3 in methanol. After extraction with petroleum ether, the fatty acid methyl esters were separated using a gas chromatograph (Model 427, Packard Instruments International, Talstrasse 39, Zurich, Switzerland) with a flame ionization detector. The chromatograph was fitted with 180 cm × 2 cm glass column packed with Chromabsorb W-AW (100–120 mesh) coated with 10% (w3w) Silar 5 CP. The proportions of the six principal fatty acids (C16:0, C16:1, C18:0, C18:1, C18:2, C20:4) were calculated on the basis of the mass of their methyl esters. Accuracy of estimation of an internal standard ranged from 0.33% to 1.5% for major fatty acids (C16:0, C18:1, C18:2) and from 1.9% to 3.3% for minor fatty acids.

Statistical analysis. Mean and standard deviation of mean were calculated by standard methods. Because the distributions of values for plasma triglycerides and HDL cholesterol were skewed, a logarithmic transformation was carried out before statistical analysis. Linear regression coefficients were calculated and used to determine relationships between the parameters. Student's t-test was used to compare the mean values, and values with $p < 0.05$ were regarded as significant.

RESULTS

As shown in Table 1, there were no significant interracial differences in the mean value of plasma cholesterol for postpartum women, nonpregnant women or neonates. The transformed mean values for the plasma triglyceride for both nonpregnant and postpartum women were significantly higher ($p < 0.005$) in the Indians than in the Negroes. However, there was no significant difference in triglycerides between Negro and Indian neonates.

The geometric mean values for the HDL cholesterol were significantly lower ($p < 0.001$) in the nonpregnant Indian women than in their Negro counterparts, but the difference in these levels in the postpartum women did not reach statistical significance. There was no difference in the values for the neonates. The ratio of HDL cholesterol to total cholesterol for the neonates was almost double the value for the women. There were no significant

TABLE 1

Plasma Lipids and Lipoprotein Concentrations in Negro and Indian Women and Neonates (Mean ± SD)

Subjects (number)	Age (yrs)	Weight (kg)	Height (cm)	Total chol. (mg/100 ml)	Log triglyc	Antilog triglyc (mg/100 ml)†	Log HDL chol	Antilog HDL chol (mg/100 ml)	HDL chol / Total chol
Nonpregnant									
Negroes (36)	26.8 ± 6.2	65.0 ± 14	162 ± 7	188 ± 40	1.91 ± 0.17	81	1.64 ± 0.11	44	0.25 ± 0.07
Indians (33)	25.5 ± 5.4	60.8 ± 16	155 ± 5	188 ± 53	2.09 ± 0.25	123	1.54 ± 0.12	35	0.22 ± 0.07
Mothers									
Negroes (37)	24.9 ± 5.6	66.4 ± 13	163 ± 7	216 ± 33	2.15 ± 0.13	142	1.68 ± 0.11	48	0.24 ± 0.05
Indians (34)	25.2 ± 5.5	65.1 ± 13	159 ± 6	210 ± 38	2.25 ± 0.13	178	1.64 ± 0.11	44	0.23 ± 0.06
Neonates									
Negroes (37)	—	3.3 ± 0.4	50 ± 4	62 ± 15	1.53 ± 0.19	34	1.39 ± 0.10	25	0.44 ± 0.08
Indians (34)	—	3.0 ± 0.4	48 ± 3	60 ± 12	1.52 ± 0.18	33	1.37 ± 0.10	23	0.42 ± 0.07

HDL, high density lipoproteins; chol, cholesterol; triglyc, triglycerides.
†Geometric mean: antilog of the mean of the log values.

TABLE 2

Plasma Apoprotein Concentrations in Negro and Indian Women and Neonates (Mean ± SD)

Subjects (number)		Age (yrs)	Apo A-I (mg/100 ml)	Apo A-II (mg/100 ml)	Apo B (mg/100 ml)	Apo A-I / Apo A-II	HDL chol / Apo A-I + Apo A-II	Total chol / Apo B
Nonpregnant								
Negroes	(36)	26.8 ± 6.2	138 ± 28	27 ± 5	98 ± 28	5.10 ± 0.89	0.25 ± 0.05	1.96 ± 0.26
Indians	(33)	25.5 ± 5.4	119 ± 28	25 ± 5	100 ± 33	4.80 ± 0.83	0.24 ± 0.04	1.87 ± 0.22
Mothers								
Negroes	(37)	24.9 ± 5.6	153 ± 27	32 ± 5	136 ± 35	4.83 ± 0.77	0.24 ± 0.06	1.73 ± 0.25
Indians	(34)	25.2 ± 5.5	154 ± 26	32 ± 6	141 ± 41	4.84 ± 0.95	0.22 ± 0.06	1.55 ± 0.19
Neonates								
Negroes	(37)	—	96 ± 18	23 ± 4	32 ± 9	4.35 ± 0.72	0.21 ± 0.05	2.02 ± 0.35
Indians	(34)	—	91 ± 17	22 ± 4	33 ± 9	4.31 ± 0.77	0.21 ± 0.05	2.04 ± 0.37

HDL, high density lipoproteins; chol, cholesterol.

differences in the values of the ratio between the adults of the two ethnic groups.

The mean apo A-I value for the nonpregnant Negro women was significantly higher ($p < 0.005$) than the mean for the nonpregnant Indian women (Table 2). This difference was absent in the postpartum women and the neonates. There were no significant differences in the value of apo A-II for the paired groups of nonpregnant women, postpartum women or neonates. Similarly, there were no significant differences in the mean value of apo B for each of the paired groups. The ratio of apo A-I to apo A-II was not significantly different in the nonpregnant and postpartum women, but this ratio was significantly lower for the neonates than the values for the adult Negro and Indian women ($p < 0.025$). Similarly, the ratio of HDL cholesterol to apo A-I + apo A-II was not significantly different in the Negroes and Indian nonpregnant and postpartum women. The value of this ratio for the neonates was significantly smaller compared with values for nonpregnant and postpartum Negro women ($p < 0.02$) and for nonpregnant Indian women ($p < 0.025$). The differences in the total cholesterol to apo B ratio between the Negroes and Indians in each group were not significant, but the mean ratios for nonpregnant women were higher than the values for postpartum women. The differences in this ratio between postpartum women and neonates were significant ($p < 0.001$).

Although dietary description was not sufficient to typify the dietary habits of the different racial groups, analysis of the five types of cooking oil used, as recorded on questionnaires, revealed that the oils were nearly identical in composition and had P/S ratios between 3.8 and 4.5.

DISCUSSION

Nonpregnant women. In the present study, it has been confirmed that in Trinidad nonpregnant Indian women in the age group of 20 to 30 yr have high plasma triglycerides and low HDL compared with values for Negro women of the same age. Until now, plasma apoproteins and cholesterol ester fatty acid composition have not been

investigated in Indians and Negroes. Indian nonpregnant women had a geometric mean plasma triglyceride value about 50% higher than Negroes, a lower HDL cholesterol geometric mean and lower plasma apo A-I and apo A-II levels. The mean plasma total cholesterol and apo B values were not significantly different. Mean values of 81 mg/100 ml for plasma triglycerides and 44 mg/100 ml for HDL cholesterol in the nonpregnant Negro women in the present study were not significantly different from the values found by Miller and Gibson (26) for Negroes in rural Caribbean communities. Our value of 135 ± 28 mg/100 ml for apo A-I in the nonpregnant Negro women is significantly higher than the value of 119 ± 28 mg/100 ml for the Indian nonpregnant women.

In agreement with other studies (21,22,27,28) the apo A-I was significantly correlated with HDL cholesterol in both Negroes and Indians, but the correlation between total cholesterol and apo B was only significant for the nonpregnant women. The values of apo B, 98 ± 28 mg/100 ml and 100 ± 35 mg/100 ml for the nonpregnant Negroes and Indians, respectively, are similar to the Lowry protein value of 100 ± 23 mg/100 ml obtained for apo B for Caucasian women of the age range 20–29 years (24). The ratio of HDL cholesterol/total cholesterol that has been suggested to be a useful factor in assessing risk of atherosclerosis (29,30) was recently shown to be related to age, sex, race and gonadal hormone use (30). In the present study, there were no significant differences in this ratio for the two ethnic groups; however, the value of this ratio was much lower than the value obtained for African women in a similar study (31).

Postpartum women. The hypertriglyceridemic and hypercholesterolemic effect of pregnancy is a well-known characteristic (32,33), but there appears to be some variation in the degree of hyperlipidemia in different communities. In the present study, the main differences between nonpregnant Negro and Indian women persisted in pregnancy, although to a lesser extent when the absolute values of triglycerides and HDL cholesterol were considered. The most prominent change in pregnancy was in the plasma triglyceride geometric value, with an increase of 75% and 45% over the values for nonpregnant Negroes and Indians, respectively, while the increase in total cholesterol, HDL cholesterol, apo A-I and apo A-II was from 9% to 29%. In a similar study (31) of African women of the same income status, a much higher increase in triglycerides, total cholesterol and HDL cholesterol was observed. The ratios of HDL cholesterol/total cholesterol, apo A-I/apo-II and HDL cholesterol/(apo A-I + apo A-II) for both pregnant and nonpregnant women were not significantly different, which suggests similarity in HDL composition. This finding confirms an earlier report of Hillman et al. (34).

The hypertriglyceridemia in pregnancy is due to an enhanced entry of triglyceride-rich lipoproteins into the circulation rather than to a diminished removal (35). This physiological adaptation would serve to make triglyceride fatty acid available to all tissue, including the placenta and the fetus. Although there is evidence to support the hormonal basis for the hyperlipidemia of pregnancy, a recent study has shown that the hypercholesterolemia in pregnancy could be ameliorated with a reduction in dietary cholesterol intake (36). A comparison of values obtained for plasma total cholesterol and triglycerides in two previous studies among Nigerian women showed a further increase in their levels during delivery compared with the value for the third trimester of pregnancy (37). This increase could be due to the effect of stress of labor during delivery.

Neonates. The cord blood samples were studied to detect any interracial differences that might exist at birth. Any such findings might suggest genetic rather than environmental causes for the differences between races. No significant differences were observed in plasma values of triglycerides, total cholesterol, HDL cholesterol, apo A-I, apo A-II or apo B, but their concentrations were below the values for the nonpregnant women. The total cholesterol values were similar to those reported by Glueck et al. (38) in their study of 1800 babies. A reported range of 54–66% of the adult apo A-I concentration in neonates (17,36) was slightly below the 71% and 76% obtained in the present study. The mean plasma HDL cholesterol in neonates was more than 50% of the nonpregnant mean, and it constituted more than 40% of the plasma total cholesterol of the neonates.

So far, there are few reports on the composition of lipoprotein classes in the cord blood. It has been shown that the composition of LDL and HDL of human umbilical cord serum is similar to that of the adult, but that the VLDL of cord blood is lower in triglyceride and higher in protein content than that from adults (39,40). It has also been shown that the composition of VLDL has no simple correlation with birth weight, sex or gestational age (40). While it is reported that the distribution of lipoproteins is different in cord blood compared with that of the adult (41), the significance of the high ratio of HDL cholesterol to total cholesterol and its decline to the adult level has not been fully investigated. It is of interest to note that the value of this ratio obtained in the present study is not different from that obtained by Taylor et al. (31) in a similar study of Africans. In the present study, the ratios of HDL to apo A-I + apo A-II and apo A-I to apo A-II for the neonates were significantly different from the values for adults, with the relative percentages of these ratios ranging from 80% to 90% of the values for the nonpregnant women.

The high prevalence of diabetes mellitus among Indians compared to Europeans (42), Negroes (10) and Africans (43) has been suggested to be the main predisposing factor in explaining the high mortality rate of CHD among Indians. However, it has been reported that diabetes mellitus was less common among Indians before the age of 25 (42) and that juvenile diabetes was also rare among Indians (43). The mean age of the Indian subjects in the present study is about 25. It is therefore probably not likely that the high prevalence of diabetes mellitus among the Indians could explain the lipid profile of this group of subjects. Moreover, no specific factor has been found to explain the high prevalence of diabetes mellitus among Indians.

In an attempt to exclude dietary factors, the plasma lipids, lipoprotein, apoprotein and cholesterol ester fatty acid composition of maternal and cord were studied, but the results did not provide any evidence of differences in lipid profile of Indians and Negroes at birth (Table 3). Thus, it appears that, as in a recent study of diet and CHD in Asian residents of London (44), the high CHD rate of the Indians in Trinidad cannot be explained by

TABLE 3

Plasma Cholesterol Ester Fatty Acid Composition in Two Racial Groups in Trinidad

Fatty acids	Maternal		Cord	
	Negroes (n = 11)	Indians (n = 9)	Negroes (n = 11)	Indians (n = 9)
C14:0	1.0 ± 0.9	0.8 ± 0.6	1.52 ± 2.3[a]	0.9 ± 1.1
C16:0	12.1 ± 1.6	12.8 ± 2.0	16.9 ± 3.5	16.7 ± 2.9
C16:1	4.0 ± 1.6	4.0 ± 1.5	6.3 ± 2.1	7.3 ± 1.9
C18:0	1.1 ± 1.2	1.5 ± 1.5	5.1 ± 1.5	4.7 ± 1.3
C18:1	17.9 ± 3.3	16.1 ± 1.2	26.3 ± 4.0	28.5 ± 7.0
C18:2	51.0 ± 7.8	52.7 ± 5.0	23.3 ± 7.6	23.1 ± 5.8
C20:4	8.7 ± 2.9	7.8 ± 2.8	18.3 ± 3.9	12.7 ± 4.4
Saturated	14.2 ± 1.3	15.2 ± 1.5	23.5 ± 2.5	22.2 ± 1.9
Monosaturated	21.9 ± 2.6	20.1 ± 1.4	32.5 ± 3.2	35.8 ± 5.4
Polyunsaturated	59.7 ± 5.6	60.5 ± 4.1	41.6 ± 6.1	40.8 ± 5.2

[a]Mean (%) ± SD.

dietary factors, plasma total cholesterol or fatty acid composition. However, the lower level of HDL cholesterol and plasma apo A-I could play a role in the higher CHD rate in the Indians.

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Membrane Fatty Acid Modification in Tumor Cells: A Potential Therapeutic Adjunct[1]

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The fatty acid compositions of several tumors have been modified sufficiently to alter some of their properties and functions. These modifications were produced in culture by adding specific fatty acids to the growth medium or by feeding fat-supplemented diets to tumor-bearing mice. The phospholipid fatty acid composition of the plasma membrane was modified, but there were no changes in membrane phospholipid or cholesterol content or in phospholipid head group composition. Each of the most abundant membrane phosphoglyceride fractions exhibited some degree of fatty acid modification. Electron spin resonance measurements with nitroxystearate spin probes indicated that the fatty acid modifications were sufficient to alter the physical properties of the plasma membrane. The K'_m for methotrexate uptake was reduced when the L1210 leukemia cells were enriched in linoleic acid. Even when the kinetics of uptake at 37 C were not altered, such as for melphalan and phenylalanine uptake, the temperature transition of transport was modified, indicating that these transport systems also are responsive to the membrane fatty acid modifications. Enrichment with highly polyunsaturated fatty acid did not affect either the growth rate or radiosensitivity of the L1210 leukemia. However, the sensitivity of the L1210 cells to the cytotoxic effects of Adriamycin and hyperthermia was increased. These findings suggest the possibility that fatty acid modification of tumors may be a useful adjunct to certain currently available therapeutic modalities.
Lipids 22, 178–184 (1987).

The fatty acid composition of cultured cells can be modified extensively by changing the type and amount of lipid contained in the growth medium (1). This is due to the fact that when fatty acids are available in the extracellular fluid, they are utilized preferentially, and de novo fatty acid synthesis is suppressed (2). Tumors also derive most of their fatty acid from the extracellular fluid when an adequate supply of lipid is available (3). Therefore, as in the case of nonmalignant cells, it seemed likely that the membrane fatty acid composition of tumor cells also might be susceptible to fairly extensive modification. We have explored this approach because of the possibility that certain lipid modifications might make a tumor more sensitive to clinically useful therapeutic modalities. This review summarizes the types of lipid modifications that we have been able to produce, both in culture and in the tumor-bearing host, and the effects of these manipulations on the properties and function of the tumor cells.

EXPERIMENTAL METHODS

The L1210 murine leukemia was grown in male DBA mice (4), and the Ehrlich ascites tumor was grown in male CBA mice (5). In the dietary modification studies, the mice were given a basal fat-deficient mixture supplemented with either 16% coconut oil (saturated fat) or 16% sunflowerseed oil (polyunsaturated fat). These diets were fed to the mice for 4 wk prior to intraperitoneal inoculation of the tumor, and the diet was continued during tumor growth (4,5). The L1210 leukemia and Ehrlich ascites carcinoma cells also were maintained in culture (6,7). Additional studies were done with cultured Y-79 retinoblastoma cells (8), Friend erythroleukemia cells (9) and hepatoma 7777 cells (10).

Transport studies were done with intact cells using radioactive isotopes and rapid separation procedures for removing the incubation medium (4,5,8). Growth rate was measured by counting the cells or following the incorporation of [^{3}H]thymidine (11). Electron spin resonance was assayed with either the 12- or 5-nitroxystearate probes incorporated into isolated plasma membrane fractions prepared from the L1210 or Ehrlich ascites cells (4,7). Phospholipids were separated by thin layer chromatography, and fatty acids were assayed by gas liquid chromatography after transmethylation (5). In the radiation sensitivity study, cells were irradiated at room temperature with a GE Maxitron x-ray machine at 250 kVp and 20 mA. The hyperthermia studies were done using a circulating water bath in which the temperature was maintained within ±0.02 C (12). Cell survival was assessed with a soft agar clonogenic assay at 37 C for 1 wk in a medium containing 20% horse serum (12).

RESULTS AND DISCUSSION

Membrane fatty acid modifications. Considerable changes in the fatty acid composition of tumor cells can be achieved, either by growth of the tumor in animals fed different fat-supplemented diets (13,14) or in culture by varying the lipids added to the medium (7,8,12). Table 1 summarizes the changes that occur in the plasma membrane of L1210 murine leukemia cells and Ehrlich ascites carcinoma cells when they are grown in mice fed a diet rich in saturated fat, as compared with polyunsaturated fat. When exposed to saturated fat, the L1210 membrane contained twice as much monoenoic, but only half as much polyenoic, fatty acid. The saturated fatty acid content was essentially unchanged. These changes were associated with a large increase in oleic acid (18:1; number of carbon atoms:number of double bonds) and a large decrease in linoleic acid (18:2). There was little or no change in the content of the other major membrane fatty acids—palmitic (16:0), stearic (18:0) or arachidonic (20:4). Similar fatty acid modifications were produced in the plasma membranes of Ehrlich ascites carcinoma cells grown in mice fed these diets. In both cases, the effects were confined to the membrane fatty acid composition, and there was no change in membrane phospholipid content, cholesterol content or phospholipid head group composition (4,15). Therefore, what occurs is a substitution of fatty acyl groups within the same phospholipid framework of the membrane.

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TABLE 1

Fatty Acid Composition of Plasma Membrane Obtained from Tumor Cells Grown in Mice Fed Diets Rich in Saturated or Polyunsaturated Fat[a]

	Membrane composition (%)[b]			
	L1210 leukemia		Ehrlich ascites carcinoma	
Fatty acid	Saturated fat[c]	Polyunsaturated fat	Saturated fat	Polyunsaturated fat
16:0	14.5 ± 0.5	14.0 ± 1.6	18.2 ± 0.2	18.9 ± 1.4
16:1	3.4 ± 0.8	2.3 ± 0.5	3.1 ± 0.3	tr[d]
18:0	23.3 ± 0.8	23.2 ± 1.0	14.4 ± 0.9	20.0 ± 1.3
18:1	24.3 ± 2.4	11.0 ± 1.7	33.1 ± 0.6	17.2 ± 1.0
18:2	8.1 ± 1.2	17.6 ± 1.6	7.7 ± 0.4	22.9 ± 1.1
20:4	8.7 ± 3.1	9.1 ± 2.2	6.6 ± 0.2	7.6 ± 0.9
22:4	0.6 ± 0.3	2.2 ± 0.2	1.2 ± 0.1	2.8 ± 0.7
22:5	3.6 ± 1.0	3.5 ± 0.2	0.8 ± 0.2	0.8 ± 0.4
22:6	1.1 ± 0.3	2.9 ± 0.1	0.6 ± 0.1	1.2 ± 0.2

[a]Cells were grown in mice fed the semipurified diet supplemented with either 16% coconut oil (saturated fat) or 16% sunflowerseed oil (polyunsaturated fat). Each value is the mean ± SE of results from three separate membrane preparations.

[b]These values do not add up to 100% because only the most prevalent fatty acids are listed.

[c]Refers to the fat contained in the diet fed to the tumor-bearing mice.

[d]Trace, <0.2%.

TABLE 2

Fatty Acid Composition of Membranes Obtained from Tumor Cells Grown in Culture

	Membrane composition (%)[a]				
	L1210 leukemia[b]		Y-79 retinoblastoma[c]		
Fatty acid	Control[d]	Docosahexaenoic acid[e]	Oleic acid[f]	Arachidonic acid[f]	Docosahexaenoic acid[f]
16:0	13.4 ± 1.8	20.0 ± 3.0	19.7	25.3	24.2
16:1	4.8 ± 0.9	3.4 ± 1.4	4.9	7.9	6.6
18:0	14.0 ± 0.4	16.7 ± 0.4	15.0	12.1	16.3
18:1	56.3 ± 1.7	25.2 ± 1.2	36.9	22.7	25.0
18:2	3.2 ± 1.2	2.3 ± 0.2	0.1	0.8	0.5
20:4	3.2 ± 1.4	1.7 ± 0.7	8.1	14.0	6.6
22:6	0.3 ± 0.2	23.4 ± 3.7	4.0	3.2	14.8

[a]Values do not add up to 100% because only the most prevalent fatty acids are listed.

[b]The growth medium contained 5% fetal bovine serum, RPMI 1640 and 40 μg/ml gentamicin sulfate. Each value is the mean ± SE of results obtained from three separate membrane preparations, each obtained from different cultures.

[c]The growth medium contained 10% fetal bovine serum, RPMI 1640, 294 μg/ml glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Each value is the average of two determinations that differed by less than 15%.

[d]No fatty acid supplement was added to the growth medium.

[e]Growth medium was supplemented with 32 μM 22:6 for 48 hr before the cells were harvested.

[f]Growth medium was supplemented with 30 μM of these fatty acids for 72 hr before the cells were harvested.

All of the major phospholipids contained in the Ehrlich cell plasma membrane were modified, although the extent of the modification was different in each fraction (15). The fatty acid compositions of the Ehrlich cell nuclear membrane and endoplasmic reticulum also were modified using this approach (16,17). Similar to these findings with ascites tumors, changes in dietary fat intake also produce alterations in the membrane lipid composition of solid mammary tumors (18–21).

Extensive changes in membrane fatty acid composition of tumor cells also can be produced by supplementing the medium with specific fatty acids during growth in culture (Table 2). When the medium of one set of L1210 leukemia cultures was supplemented with 32 μM docosahexaenoic acid (22:6), there was a 250% elevation in the polyenoic fatty acid content of the membrane, accounted for primarily by an increase in the 22:6 content. This was compensated for by a 57% decrease in monoenoic fatty acids, primarily 18:1. Enrichment with 22:6 also was associated with a 35% increase in the membrane saturated fatty acid content.

Similar modifications in membrane fatty acid composition have been produced in cultured human Y-79 retinoblastoma

cells (1,8). Table 2 illustrates the differences in membrane fatty acid composition that occurred when the medium in which the Y-79 cells are grown was supplemented with 30 μM of oleic, arachidonic or docosahexaenoic acid. As noted in the L1210 membranes, the saturated fatty acid content of the membranes increased to a small extent when the Y-79 cells were enriched with the polyunsaturated fatty acids. Compared to membranes from cells supplemented with oleic acid, those grown in the medium supplemented with arachidonic acid contained considerably less 18:1 and 73% more 20:4. Likewise, the membranes from cells supplemented with docosahexaenoic acid contained less 18:1 and 270% more 22:6 than those from the cells supplemented with oleic acid.

These types of membrane fatty acid modifications also have been produced in cultured Ehrlich ascites carcinoma cells (7), Friend erythroleukemia cells (9) and hepatoma 7777 cells (10). The advantage of this method, as opposed to the use of fat-supplemented diets, is the enrichment with the higher polyunsaturates such as 20:4 and 22:6 that can be produced in culture.

Membrane physical properties. Using electron spin resonance with fatty acid spin probes, we find that the physical properties of the plasma membrane lipids are altered by these kinds of phospholipid fatty acid replacements. Arrhenius plots of temperature dependence of the approximate rotational correlation time with nitroxystearate spin probes indicated that the membranes have two major temperature transitions, one at about 20 C and the other at 31.5 C. Results obtained with L1210 leukemia and Ehrlich ascites carcinoma plasma membranes are consistent (4,7,22). Changes in the fatty acid composition of the plasma membrane had essentially no influence on the 31.5 C transition, but they caused the lower transition to vary from 18.5-22 C in the L1210 plasma membrane and from 19-24.5 C in the Ehrlich membrane when the modifications were produced by feeding different fats to the tumor-bearing mice (4,22). In agreement with these findings, fatty acid substitutions in culture caused the lower transition in the Ehrlich ascites cell plasma membrane to vary between 20.5 C and 26 C (7). Enrichment with polyenoic fatty acids lowered this transition temperature, while higher values were obtained with saturated fatty acids.

The electron spin resonance order parameter, S, also was influenced by membrane fatty acid modifications produced either by diet or in culture (4,7,22). This parameter is a measure of phospholipid fatty acyl chain mobility; hence, the packing of these hydrocarbon chains in the lipid bilayer. The effect of fatty acid modification of the L1210 plasma membrane was similar with both the 5- and 12-nitroxystearate probes (Fig. 1). The value of S was lower at each temperature when the cells were grown in the mice fed the diet rich in polyunsaturated fat, which increased the polyenoic fatty acid content of the L1210 membrane phospholipids. Decreases in the plasma membrane S values also were noted when Ehrlich ascites carcinoma and L1210 leukemia cells were enriched with polyunsaturated fatty acids in culture (7,12,22). Further work indicated that the changes in S values were not due to the trapping of cytoplasmic lipid droplets in these membrane preparations (23).

Throughout the temperature range tested, the value of S was always higher with the 5-nitroxystearate compared

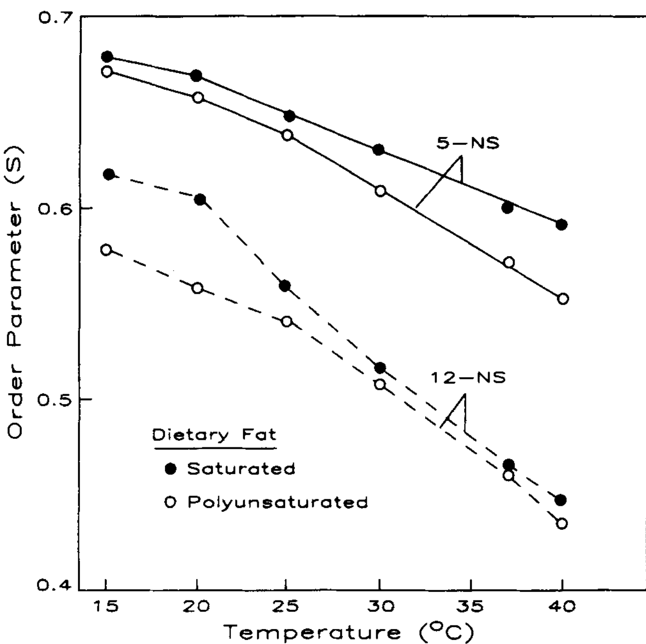

FIG. 1. Effect of changes in plasma membrane saturation of L1210 leukemia cells on the order parameter (S) measured by electron spin resonance. One group of cells was grown in mice fed the saturated diet, the other the polyunsaturated fat diet. After the plasma membrane fraction was isolated from the cell homogenate, the spin label was incorporated into the membrane by incubation with a solution containing 5% serum albumin. The abbreviations refer to the spin probes that were incorporated into the membranes: 5-NS, 5-nitroxystearate; 12-NS, 12-nitroxystearate.

to the 12-nitroxystearate probe (Fig. 1). It is assumed that these fatty acid spin probes enter the membrane lipid bilayer and are oriented parallel to the phospholipid fatty acid chains. In such orientation, the fatty acid carboxyl group would be at the surface of the phospholipid bilayer in the same plane as the polar head groups of the phospholipids, and the 5-nitroxide group would be located closer to the membrane surface than the 12-nitroxide group. The higher S value with the 5-nitroxystearate probe suggests that fatty acyl chain motion is more restricted closer to the membrane surface, near the ester linkage with the phospholipid head group, than deeper with the hydrocarbon phase.

These findings suggest that the lipid modifications produced in the intact tumor cell are sufficient to alter the physical properties of the plasma membrane lipid bilayer, i.e., to produce a membrane fluidity change. Since neither the cholesterol nor phospholipid content of the membrane changes, the fluidity effect results entirely from the fatty acid modifications. Because the differences in the values of S at 37 C are very small, the physiologic effects of the fluidity changes probably are fairly limited.

Carrier-mediated transport. To determine whether the lipid modifications actually can have an effect on membrane function, we investigated the uptake of a number of substances that are transported by carrier-mediated processes. As shown in Table 3, changes were noted in the K'_m of high-affinity uptake in several cases. The K'_m for the uptake of methotrexate, a chemotherapeutic drug, was reduced by 30% at 37 C when L1210 leukemia cells

TABLE 3

Effect of Fatty Acid Modification on the K'_m for High-affinity Transport[a]

Compound	Cell	K'_m (μM) Saturated fat	Polyunsaturated fat
Methotrexate	L1210	4.1 ± 0.1	2.9 ± 0.4
Melphalan	L1210	0.9 ± 1.8	1.2 ± 0.5
α-Aminoisobutyrate	Ehrlich	2300 ± 200	900 ± 200
Phenylalanine	Ehrlich	120 ± 30	140 ± 100
Choline	Y-79	2.2 ± 0.1	1.1 ± 0.2
Taurine	Y-79	37 ± 6	20 ± 1
Glycine	Y-79	34 ± 3	136 ± 27
Glutamate	Y-79	27 ± 1	28 ± 6
Leucine	Y-79	95 ± 19	72 ± 8

[a]The murine L1210 leukemia cells and the Ehrlich ascites carcinoma cells were modified by diet as indicated in Table 1. The human Y-79 retinoblastoma cells were modified in culture using a growth medium containing 10% fetal bovine serum. No fatty acid supplement was added to the growth medium of the Y-79 cultures for the column headed "saturated fat." In the case of the column headed "polyunsaturated fat," the growth medium of the Y-79 cultures was supplemented with 30 μM 22:6. Each value is the mean ± SE of results obtained from at least four separate experiments.

were enriched with polyunsaturated fatty acid by propagation in mice fed the diet supplemented with sunflowerseed oil (4). In Ehrlich cells, enrichment with polyunsaturated fatty acid in this way resulted in a 60% reduction in the K'_m for Na^+-dependent α-aminoisobutyrate uptake at 37 C (5). When human Y-79 retinoblastoma cells were enriched with polyunsaturated fatty acids in culture, the K'_m for choline and taurine transport at 37 C also was lowered (8,24), but that for glycine was increased (25). Table 3 shows the results for the Y-79 cells enriched with 22:6; qualitatively similar changes were obtained when the cells were enriched in culture with other polyunsaturated fatty acids (8,24,25).

As opposed to these positive findings, no statistically significant change in the high-affinity K'_m at 37 C was observed with melphalan (a phenylalanine mustard alkylating agent) in L1210 cells (26), phenylalanine in Ehrlich cells (27) or glutamate and leucine in Y-79 cells (8). In two of these cases, however, melphalan in L1210 cells and phenylalanine in Ehrlich cells, the transition temperature of the transport system was lower when the cells were enriched with polyunsaturated fatty acid (26,27).

No statistically significant change in V'_{max} occurred in those cases where the K'_m for transport at 37 C was reduced—methotrexate in L1210 cells (4), α-aminoisobutyrate in Ehrlich cells (5) or choline and taurine in Y-79 retinoblastoma cells (8,24). By contrast, the V'_{max} for glycine transport was increased three- to fivefold when the Y-79 retinoblastoma cells were enriched with polyunsaturated fatty acids (25).

These findings indicate that a number of carrier-mediated transport systems in several kinds of tumor cells, including a human tumor, are affected by the types of membrane fatty acid modifications that can be produced in the intact cell. Since the K'_m of the transport process is altered, the mechanism probably involves lipid structural effects on the conformation of the membrane carrier that are sufficient to influence the binding of the substrate. The effects on transport kinetics at 37 C, however, are not uniform for all substances, suggesting that the mechanism is more complex than a general response to changes in bulk membrane fluidity. Even in those cases in which no kinetic changes are evident at 37 C, the carrier appears to be sensitive to the membrane lipid microenvironment, as evidenced by the changes in the transition temperature.

Growth rate. Studies with the L1210 leukemia indicate that the growth of the tumor is not changed significantly when the cells are enriched with polyunsaturated fat in vivo, as compared to saturated fat (11). Cell counts obtained on days 5, 6 and 7 following intraperitoneal inoculation of the tumor were not different in the two dietary conditions. Likewise, [^{3}H]thymidine incorporation by the cells was unchanged. Consistent with our findings, dietary fat modifications also had no effect on the growth of the MT-W9B rat mammary tumor (28). These results suggest that there is unlikely to be any direct beneficial effect of these types of lipid modifications on tumor growth. Therefore, we directed our attention to the possibility that the lipid modifications may facilitate other treatment modalities.

Adriamycin cytotoxicity. Studies on lipid composition of Adriamycin-resistant P388 cells (P388/Adr) have revealed a decrease in the phosphatidylcholine to sphingomyelin ratio (29) and a higher degree of membrane lipid structural order (30). Similarly, studies on a series of sublines of Sarcoma 180 with different degrees of sensitivity to Adriamycin showed a correlation between drug resistance and membrane fluidity (31). These observations suggest that membrane lipids may have important implications regarding Adriamycin therapy. Therefore, we examined the effects of lipid modifications on the sensitivity of the L1210 and P388/Adr tumor cell lines to Adriamycin cytotoxicity.

L1210 cell survival in culture after treatment with Adriamycin was reduced when the cells were enriched with polyunsaturated fatty acids (6). Most of this work was done with 22:6 enrichment to maximize the increase in polyunsaturation. Increased cytotoxicity occurred at all of the Adriamycin concentrations tested (Fig. 2). In this study the cells were grown for 48 hr in either control medium or a medium supplemented with 32 μM 22:6. After removal of this medium, the cultures were exposed to various concentrations of Adriamycin for 2 hr, and the percentage of surviving cells was determined by a clonogenic assay based on colony formation in soft agar (6). Additional studies indicated that the amount of Adriamycin cytotoxicity increased as increasing quantities of 22:6 were incorporated into the cell lipids (6). The clonogenic assay measures the ability of single cells to generate daughter cells for more than 2-3 mitotic divisions. This is an appropriate measure of the cytotoxic effect of an antineoplastic therapy, since it is the reproduction of surviving cells that perpetuates the tumor.

The augmented cytotoxicity is due to an enhanced effect of Adriamycin toward 22:6-enriched L1210 cells and is not simply the result of greater polyunsaturation alone. We have previously demonstrated that the culture doubling time for L1210 cells grown in 22:6-supplemented medium is 10.0 ± 0.3 hr, which is not different from that

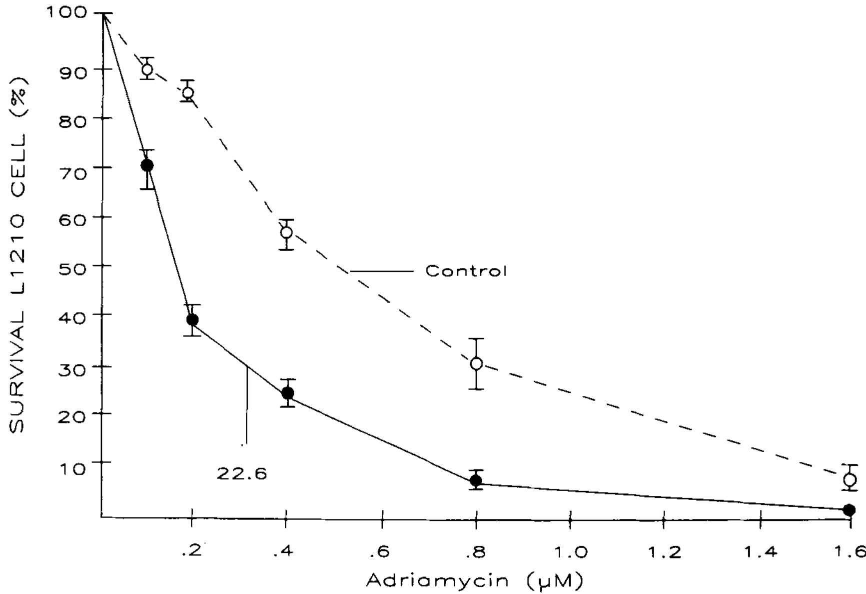

FIG. 2. Effect of phospholipid fatty acid saturation on the cytotoxicity of Adriamycin in L1210 leukemia cells. The growth medium contained RPMI 1640 and 5% fetal bovine serum. In one set of cultures, the medium was supplemented for 48 hr with 32 μM 22:6. Each point is the mean ± SE of 4 determinations.

of L1210 cells grown in unsupplemented medium, 9.2 ± 0.9 hr (6). Likewise, the soft agar cloning efficiency of cells enriched in 22:6 is not different (6).

The enhanced cytotoxicity was associated with an increase in the accumulation of Adriamycin in the L1210 cells (32). No difference in Adriamycin release from the cells occurred, however, indicating that the greater accumulation did not result from an inhibition of Adriamycin efflux. Binding and lipid partitioning studies also indicated that the greater intracellular accumulation is not due to increased Adriamycin adsorption to the polyunsaturated fatty acid-enriched membranes. At present, the mechanism responsible for the greater accumulation is unknown.

We have also investigated whether polyunsaturated fatty acid enrichment would confer Adriamycin sensitivity to a tumor that normally was resistant to this drug, the murine P388/Adr lymphoma. P388/Adr cells were enriched in culture with either 32 μM 18:1 or 22:6 and then exposed to 0.4 μM Adriamycin for up to 4 hr (Fig. 3). No cytotoxicity differences were observed in a subsequent assay, indicating that a large increase in polyunsaturation will not cause an otherwise resistant tumor cell to become Adriamycin-sensitive.

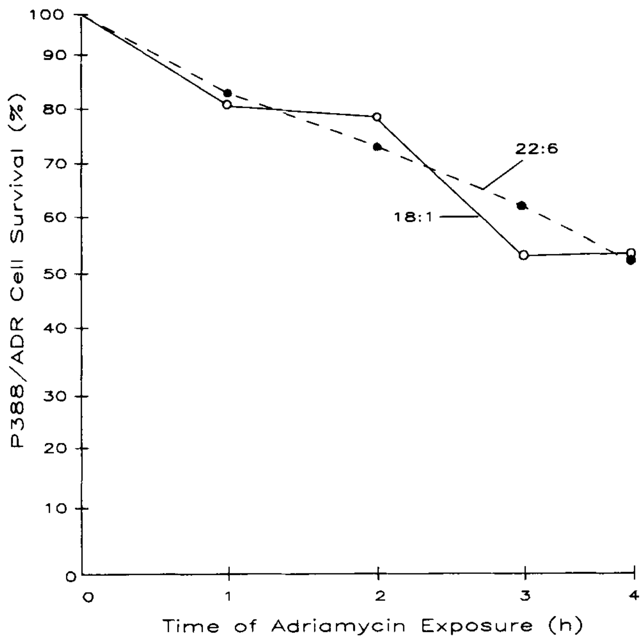

FIG. 3. Effect of changes in phospholipid fatty acid saturation on the cytotoxicity of Adriamycin in P388/Adr lymphoma cells. The growth medium of the cultures was enriched with either 18:1 or 22:6.

Radiation sensitivity. Polyunsaturated fatty acid enrichment did not affect the sensitivity of the L1210 leukemia cells to ionizing radiation (33). L1210 cells grown in mice fed the diets rich in either saturated or polyunsaturated fat were irradiated with increasing doses of x-rays, and cytotoxicity was assessed subsequently with a soft agar clonogenic assay. No difference in cytotoxicity was observed (Fig. 4). A similar negative result was obtained when the polyunsaturation of the human Y-79 retinoblastoma cell line was raised in culture by supplementation of the medium with 22:6.

Sensitivity to hyperthermia. There is considerable evidence documenting the cytotoxic effect of elevated temperatures on tumors in vitro (34) and in vivo (35). In our studies, the thermosensitivity of the L1210 leukemia increased when the cells were enriched with polyunsaturated fatty acid (Fig. 5). In this study, the cultures were grown in media containing 40 μM of the supplemental fatty acid for 10 days, removed from this medium and then heated at the designated temperatures. Cytotoxicity was assessed by a soft agar clonogenic assay (12). At 42 C, the D_0 values (minutes of heating required to reduce

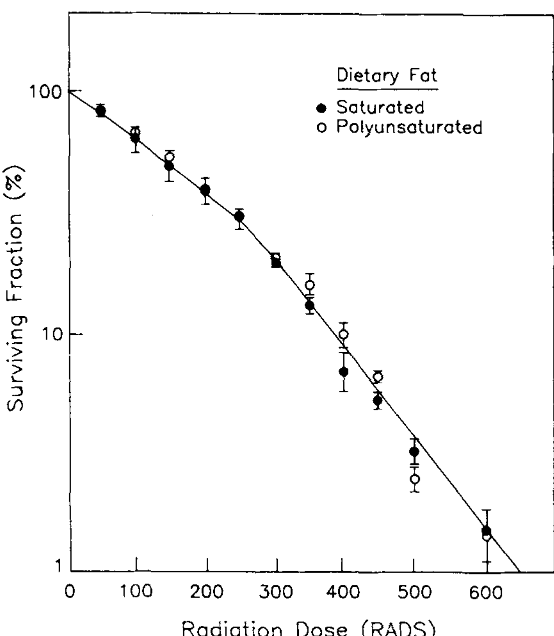

FIG. 4. Effect of changes in phospholipid fatty acid saturation on the cytotoxicity of x-ray exposure in L1210 leukemia cells. The cells were grown in mice fed either the saturated or polyunsaturated fat diets. Each point is the mean ± SE of 3 determinations.

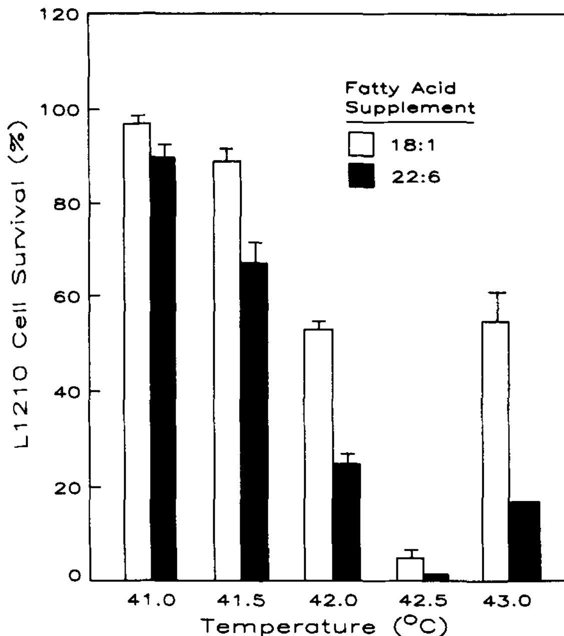

FIG. 5. Effect of changes in phospholipid fatty acid saturation on the thermosensitivity of L1210 leukemia cells. The growth medium of the cultures contained 5% fetal bovine serum and was supplemented with either 40 μM 18:1 or 22:6. Each bar is the mean ± SE of 4 determinations. Cells were heated for 90 min at the temperatures of 41.0-42.5 C. At 43 C, no cell survival was detected at 90 min; the study therefore was done at 30 min.

cell survival on the exponential portion of the curve by 63%) for cells enriched with 18:1 and 22:6 were 46.2 ± 1.6 min and 18.7 ± 0.4 min, respectively. Additional studies in which the cultures were heated for 2 hr at 42 C indicated that thermosensitivity increased as the 22:6 content of the cell lipids was raised (12). Therefore, changes in fatty acid saturation cause the L1210 cell to respond differently to various kinds of radiation. Although the response to ionizing radiation is not affected, the sensitivity to thermal radiation becomes enhanced.

Conclusions. These findings demonstrate that it is possible to change the fatty acid composition of an intact tumor cell sufficiently to alter the physical properties of the plasma membrane and to affect certain cellular responses. The growth rate of the tumor in vivo is not affected by these changes. This suggests that if such lipid modifications have any therapeutic benefit, it probably is as an adjunct to other treatment modalities rather than as an independent form of treatment. Since functionally significant lipid modifications can occur as the result of changing the dietary fat intake of a tumor-bearing host, the possibility of applying this approach to humans is feasible. Moreover, for localized tumors, it may be possible to produce specific fatty acid modifications such as those that have been achieved in cell culture by using a localized perfusion technique.

The diets that were used alter the lipid composition of many organs in the mouse (36). Therefore, the changes are not tumor-specific, raising the possibility that this approach may also increase the toxicity of some therapeutic modalities. Another concern is that high fat diets, particularly those that are rich in essential polyunsaturated fatty acids, enhance the development of certain tumors (37-43), apparently by facilitating tumor promotion (44-46). While these concerns must be considered, the strikingly positive responses obtained with Adriamycin and hyperthermia suggest that the approach of utilizing membrane lipid modification in conjunction with existing forms of therapy merits further exploration.

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Biochemical Characterization of Acetyl-CoA:1-Alkyl-2-Lyso-*sn*-Glycero-3-Phosphocholine Acetyltransferase in Rat Spleen Microsomes

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Acetyl-CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF) ultrasonic disruption in the presence of 25% glycerol from rat spleen microsomes. About 26% of the enzymatic activity was recovered in the 225,000 × g supernatant by this treatment, although the specific activity was slightly decreased compared with the original microsomes. The solubilized enzyme was remarkably susceptible to various kinds of metal ions. Sulfhydryl reagents such as p-chloromercuribenzoate and N-ethylmaleimide significantly inhibited the enzyme reaction, suggesting that the enzyme is an SH enzyme. Based on the sedimentation pattern in sucrose density centrifugation, the isoelectric point, the kinetic characteristics and the sensitivity to tryptic digestion of microsomes, it appears that acetyl-CoA:lyso-PAF acetyltransferase does not differ from the acetyltransferase responsible for the transfer of acetate from acetyl-CoA to 1-acyl-2-lyso-*sn*-glycero-3-phosphocholine.
Lipids 22, 185–189 (1987).

Platelet-activating factor (PAF) is a potent phospholipid mediator capable of promoting numerous biological effects, including platelet activation, hypotension and bronchoconstriction (1,2). Neither the 1-acyl-linked analog nor the nonacetylated analog was active at comparable doses of PAF (3–5). Specific enzymatic reactions involved in the biosynthesis of PAF have been documented in recent reports. These reactions include an acetyl-CoA:1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF) acetyltransferase (EC 2.3.1.67) (6–8) and a CDP-choline:1-alkyl-2-acetyl-glycerol cholinephosphotransferase (9). Both enzymatic activities are present in various rat tissues (6,9), human polymorphonuclear leucocytes (8,10), murine macrophages (11–13) and rabbit platelets (14). However, the activity of acetyltransferase is induced up to 10-fold when polymorphonuclear leucocytes are treated with opsonized zymosan (10). Under these conditions, the activity of cholinephosphotransferase is not affected (10). Furthermore, modulation of acetyltransferase activity by treatment of various cell types with different factors is accompanied by a parallel change in the amount of PAF released into the media (11,13,15). These results suggest that acetyltransferase is an important regulatory enzyme in the biosynthesis of PAF. It is not yet known how various stimuli regulate the biosynthesis of PAF.

Wykle et al. (6) found that a significant amount of acetyltransferase activity occurred in the microsomal fraction of the rat spleen. However, actual attempts to remove the enzyme from its membrane environment have not been performed. This paper describes for the first time an effective procedure for solubilization and partial characterization of acetyltransferase. In addition, we have investigated whether the enzyme reactions introducing the acetyl moiety into the c-2 position of lyso-PAF and 1-acyl-2-lyso-*sn*-glycero-3-phosphocholine (LPC) are catalyzed by the same enzyme.

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MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following commercial sources: acetyl-CoA, p-chloromercuribenzene sulfonic acid (p-CMBS), phenylmethylsulfonic acid (PMSF), trypsin (bovine pancreas), trypsin inhibitor (soybean) and phospholipase C *(Bacillus cereus)* were from Sigma (St. Louis, Missouri); diisopropyl fluorophosphate (DIPF) was from Fluka (Tokyo, Japan); lyso-PAF (1-O-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine) was from Bachem Feinchemikalien (Budendorf, Switzerland); and LPC (1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine) was from Nippon Shoji (Osaka, Japan). PAF was prepared semisynthetically from rat fish *(Hydrolagus colliei)* liver oil as described previously (16). The chemical 1-O-hexadecyl-2-acetyl-*rac*-glycerol was synthesized starting from 1-O-hexadecyl-*rac*-glycerol (chimyl alcohol) (17,18). N-ethylmaleimide (NEM) was obtained from Wako (Tokyo, Japan); carrier Ampholite (pH 3.5–10) was from LKB (Uppsala, Sweden); and [1-^{14}C]acetyl-CoA (55 Ci/mol) was from Amersham (Arlington Heights, Illinois). The specific activity was adjusted to 2.5 Ci/mol by the addition of unlabeled acetyl-CoA. All other chemicals were reagent grade.

Preparation of microsomes. Spleens were excised from Wistar rats (200–300 g) after they were killed by decapitation. Microsomal fractions were prepared according to the procedure described by Lenihan and Lee (19) and were suspended in 0.25 M sucrose/20 mM Tris-HCl buffer (pH 7.4)/1 mM dithiothreitol (10–15 mg of protein/ml).

Enzyme assay. Acetyl-CoA:lyso-PAF acetyltransferase activity was determined by the method described by Wykle et al. (6), with several modifications. The incubation mixture consisted of 100 μM [1-^{14}C]acetyl-CoA, 30 μM lyso-PAF or LPC, 0.1 M potassium phosphate buffer (pH 6.9) and enzyme protein (20–100 μg) in a final volume of 0.25 ml. After incubation for 10 min at 37 C, the enzymatic reaction products were extracted by the method of Bligh and Dyer (20). The radioactivity was measured by liquid scintillation counting. Results were collected with backgrounds without added lyso-PAF or LPC.

Analytical methods. Protein was determined by either the method of Bensadoun and Weinstein (21) or the Bio-Rad protein assay kit, using bovine serum albumin as a standard.

RESULTS

Solubilization of acetyltransferase. In an effort to solubilize acetyltransferase from rat spleen microsomes, we first tried various detergents, including Triton X-100,

TABLE 1

Solubilization and Partial Purification of Acetyltransferases from Rat Spleen Microsomes

Procedure	Protein (mg)	Total activity (nmol/min)		Specific activity (nmol/min/mg)		Yield (%)	
		Lyso-PAF	LPC	Lyso-PAF	LPC	Lyso-PAF	LPC
Microsomes	23.8	38.5	35.6	1.62	1.50	100	100
Solubilization	8.8	10.2	6.3	1.16	0.72	26.5	17.7
Sepharose 6B	3.0	8.3	5.9	2.74	1.96	21.6	16.6

Lyso-PAF, 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; LPC, 1-acyl-2-lyso-*sn*-glycero-3-phosphocholine.

Tween 80, octylglucoside, Lubrol, Briji, Miranol HM2 and cholate derivatives. However, the enzyme activity was remarkably inhibited by these detergents in spite of their low concentration (below 0.2%). Finally, we were able to solubilize the enzyme using the following steps: Glycerol (a final concentration of 25%) was added to the microsomal suspension, and the mixture was subjected to an ultrasonic disruption in a Branson sonifier equipped with a microtip at 0 C for three 1.5-min periods. The mixture was centrifuged at 225,000 × g for 1 hr, and the resultant supernatant was the source of the solubilized enzyme, in which ca. 26% of the acetyltransferase activity was recovered (Table 1). Then the solubilized enzyme was applied to a Sepharose 6B column (1.5 × 75 cm) previously equilibrated with 0.25 M sucrose/10 mM potassium phosphate buffer (pH 7.4)/1 mM dithiothreitol/10% glycerol. The active fractions eluting near the void volume were pooled and used in all the subsequent experiments as partially purified enzymes. Enzymes were partially purified ca. 1.7-fold with a 21% yield. The final enzyme preparation was extremely labile, and ca. 50% of the activity was lost after 24 hr at 4 C.

On the other hand, rat spleen microsomes also catalyzed the transfer of acetate to LPC. The LPC acetyltransferase activity was solubilized and partially purified by the same procedures used for lyso-PAF acetyltransferase. The results were similar to those obtained with lyso-PAF acetyltransferase.

Enzymatic properties of lyso-PAF acetyltransferase and LPC acetyltransferase: sucrose density gradient centrifugation. Both lyso-PAF and LPC acetyltransferases appeared in almost the same fraction near the top of the centrifuge tube, although lyso-PAF acetyltransferase activity showed a shoulder on the right-hand side (Fig. 1).

Isoelectric focusing. The isoelectric point of lyso-PAF and LPC acetyltransferase was a single peak at pH 4.25 (Fig. 2).

Effect of various chemicals. As shown in Table 2, there was no significant difference in the effects of various reagents on either form of acetyltransferase. Both EDTA and EGTA inhibited the enzyme activities, but the inhibition did not appear related to a divalent metal ion requirement. Divalent cations inhibited the activity of both forms of transferase to various degrees. Mn^{2+} showed an especially striking inhibition. On the other hand, p-CMBS and NEM remarkably inhibited the enzyme activity, indicating that the enzymes are SH enzymes. DIPF and PMSF, however, had no effect.

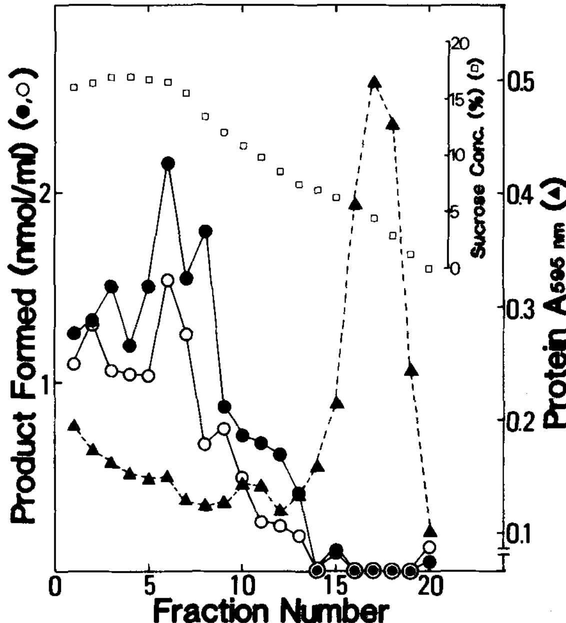

FIG. 1. Sucrose density gradient centrifugation. The solubilized enzyme (4.8 mg of protein) was applied to the top of a linear sucrose gradient (0–20%) in a 50-mM potassium phosphate buffer (pH 7.4)/1 mM dithiothreitol/10% glycerol that was prepared in a 10-ml centrifuge tube and centrifuged at 11,700 × g for 14 hr at 4 C using a Hitachi RPS-40T rotor. After centrifugation, fractions of 555 μl each were collected from the bottom of the tube. The protein was determined in 10 μl of an aliquot using the Bio-Rad protein assay kit. ●, Lyso-PAF acetyltransferase; ○, LPC acetyltransferase; ▲, protein. Sucrose concentrations (□) were measured by a refractometer.

Kinetics. The transferase activity was linear with protein concentrations up to 50 μg and throughout 25 min of incubation (data not shown). The optimum concentrations of both lyso-PAF and LPC were ca. 50 μM (Fig. 3). Lyso-PAF or LPC concentrations above 50 μM reduced the enzyme reactions strikingly, possibly due to the detergent effects of lysophospholipids (6,23). An apparent Km value for acetyl-CoA was the same (196 μM) with both lyso-PAF and LPC acetyltransferase.

Effect of tryptic digestion of microsomes. The enzyme activity in the intact microsomes was susceptible to tryptic digestion, and lyso-PAF and LPC acetyltransferase activity were inactivated in a parallel manner in relation to incubation time (Fig. 4).

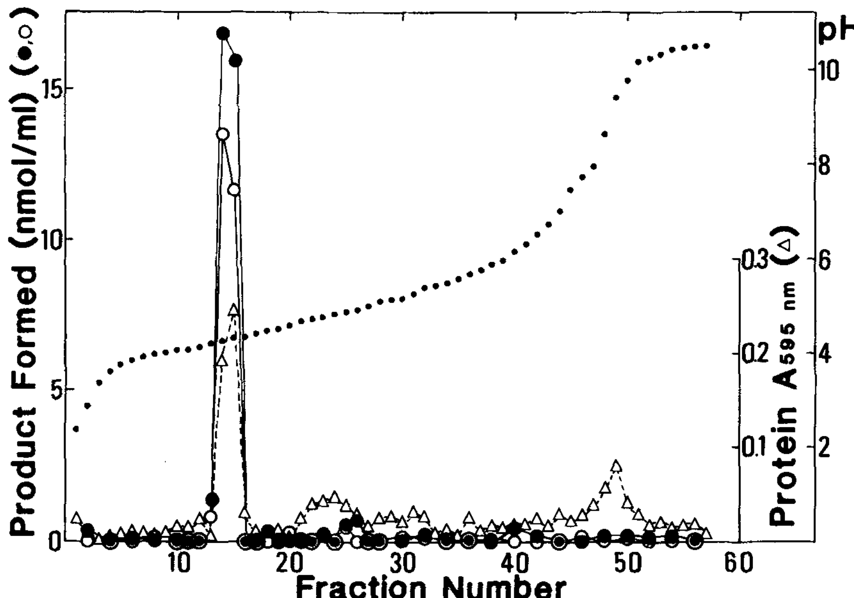

FIG. 2. Isoelectric focusing. The solubilized enzyme (2.8 mg of protein) was subjected to a column (110 ml) containing 1% Ampholine carrier ampholyte solution, over a pH range of 3.5-10, with a step-wise sucrose gradient at 500 V for 48 hr, according to the method of Vesterberg (22). The column content was collected separately in 1-ml fractions each at a flow rate of 80 ml/hr. The pH of fractions was measured in an ice-cold bath. Protein was determined in 10 μl of an aliquot using the Bio-Rad protein assay kit. ●, Lyso-PAF acetyltransferase; ○, LPC acetyltransferase; △, protein.

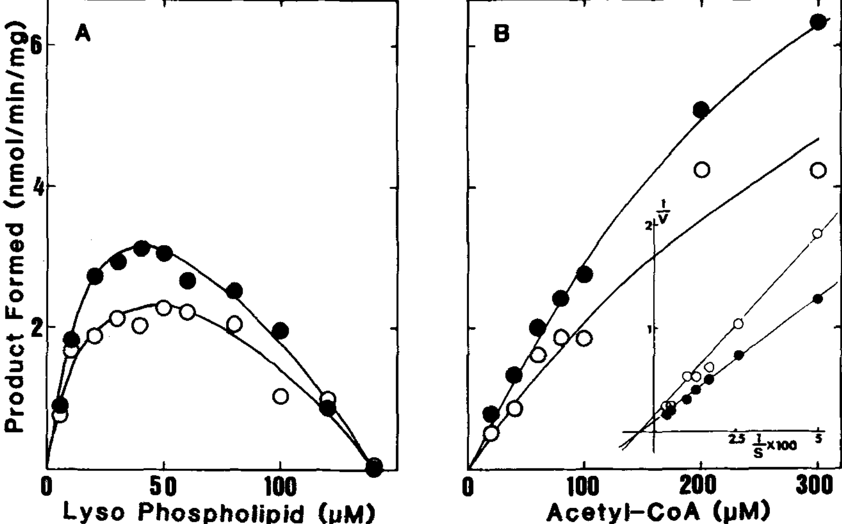

FIG. 3. Kinetic studies. Enzymatic activity was determined as described in Materials and Methods, except that the substrates were changed as indicated and 24 μg of protein was used. A, Effect of lysophospholipid concentration; B, effect of acetyl-CoA concentration; ●, lyso-PAF acetyltransferase; ○, LPC acetyltransferase.

Product identification. The following steps were carried out to identify the reaction product: (a) The reaction product was hydrolyzed with phospholipase C and acetylated with acetic anhydride. The chemically modified radioactive product was chromatographed by TLC. The reaction product had exactly the same mobility as that of authentic 1-alkyl-2,3-diacetyl glycerol when lyso-PAF was used as a substrate (Fig. 5). When LPC was used as a substrate, on the other hand, 1-acyl-2,3-diacetyl glycerol was the major reaction product. However, a small amount of reaction product was detected even though lysophospholipids were not added exogenously, which suggests that either endogenous lyso-PAF or LPC may be used as an acetyl acceptor. (b) The reaction product elicited a significant platelet aggregation in a dose-dependent manner when lyso-PAF was added as a substrate (Fig. 6).

TABLE 2

Effect of Various Reagents on Acetyltransferase Activities

Reagents added	Concentration (mM)	Relative activity (%)	
		Lyso-PAF	LPC
None	—	100	100
EDTA	1	14	12
EGTA	1	28	22
$CaCl_2$	10	42	44
$MgCl_2$	10	76	76
$MnCl_2$	10	4	6
NaCl	200	29	36
KCl	200	26	33
p-CMBS	0.5	0	0
NEM	1	11	14
DIPF	1	96	117
PMSF	5	116	92

Lyso-PAF, 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; LPC, 1-acyl-2-lyso-*sn*-glycero-3-phosphocholine; p-CMBS, p-chloromercuribenzene sulfonic acid; NEM, N-ethylmaleimide; DIPF, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride.

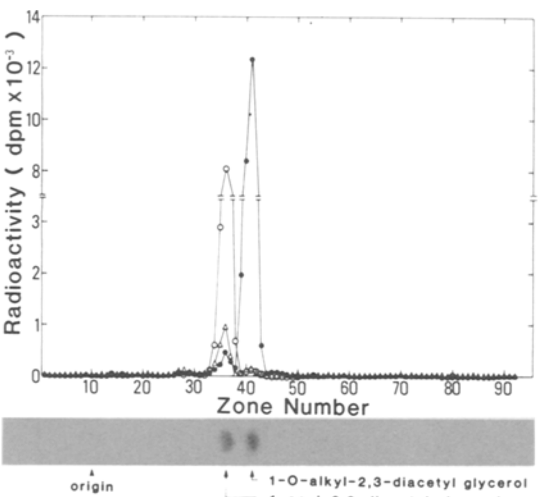

FIG. 5. Thin layer chromatographic identification of the reaction product. The enzymatic reaction products of several incubations with identical substrates were pooled and applied to thin layer plate developing with chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v). Radioactive products with the same mobility as authentic PAF were extracted and hydrolyzed with phospholipase C (28), followed by acetylation with acetic anhydride (29). The reaction product was chromatographed on a thin layer plate, which first was developed 7 cm from the origin with hexane/ethyl ether/acetic acid (50:50:1, v/v/v) and then 15 cm from the origin with toluene. The silica gel plate was scraped in 2-mm sections, and the radioactivity was determined by liquid scintillation counting. ●, Lyso-PAF acetyltransferase; ○, LPC acetyltransferase; △, no lysophospholipid added.

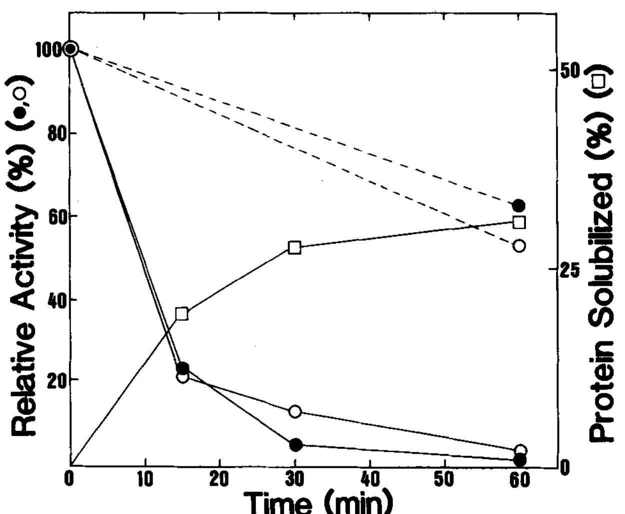

FIG. 4. Effect of tryptic digestion of rat spleen microsomes. Microsomes (10 mg of protein) were digested with trypsin (5 mg) at 30 C, and the reaction was stopped at the indicated time by adding a trypsin inhibitor (7.5 mg). The trypsin-treated microsomes were isolated by centrifugation at 105,000 × g for 1 hr. ●, Lyso-PAF acetyltransferase; ○, LPC acetyltransferase; □, protein solubilized. Dotted line represents an untreated control.

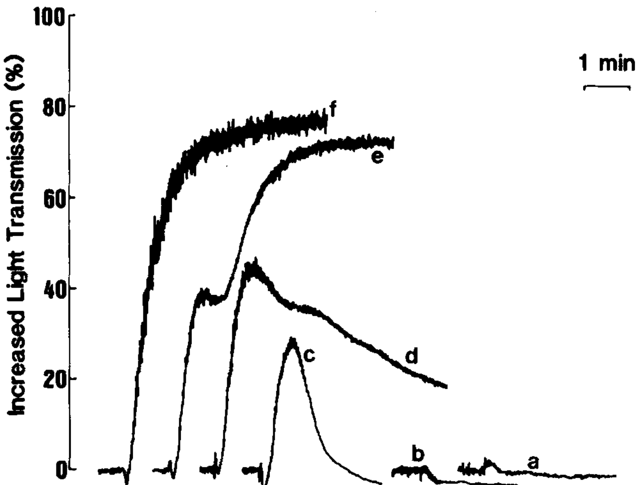

FIG. 6. Platelet aggregation by the enzyme reaction product. The radioactive product was pooled as described in Fig. 5, and the biological activity was assessed by platelet aggregation (30). Human venous blood was drawn from healthy volunteers and added to 1/10 volumes of 3.8% citrate anticoagulant. Platelet-rich plasma (PRP, $5 \times 10^5/mm^3$) was prepared by centrifugation at 250 × g for 15 min. Platelet aggregation was measured by the turbidimetric method using an aggregometer (31). The reaction was started by the addition of the sample to 200 μl of PRP maintained at 37 C with continuous stirring. The radioactive product was prepared from an incubation mixture containing the following lysophospholipids as substrates: None (a), LPC 476 nM (b), lyso-PAF 48 nM (c), 91 nM (d), 176 nM (e) and 244 nM (f).

However, the reaction product showed no platelet aggregation when LPC was used as a substrate.

DISCUSSION

Solubilization of membrane-bound enzymes without loss of activity has been achieved in the past by the use of nondenaturating detergents such as Triton X-100. The acetyltransferase under investigation here, however, was completely inactivated by these detergents. One possible explanation for such inactivation is that the detergents replace membrane-phospholipids and that the association

of these membrane-phospholipids with the enzyme may be essential for enzyme activity. In these experiments, we were able to solubilize effectively the acetyltransferase from rat spleen microsomes by ultrasonic disruption in the presence of 25% glycerol. However, it is possible that the activity is still associated with small membrane vesicles that failed to sediment. By a similar procedure, phosphatidylglycerol phosphatase (EC 3.1.3.27) was solubilized from rat liver mitochondria (32).

Wykle et al. (6) reported the presence of an acetyltransferase activity in rat spleen microsomes. The product of the reaction was identified by TLC, but its biological potency in platelet aggregation tests was not documented. Satouchi et al. (33) reported that acyl-type PAF, 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine, was produced concomitantly with PAF by stimulated rabbit polymorphonuclear neutrophiles. This correlation may suggest that the enzymes involved in the steps of deacylation and acetylation in PAF synthesis (34) do not have strict selectivity for linkage at the *sn*-1 position of choline phosphoglyceride. In fact, an acyl analog of lyso-PAF, LPC, can serve as a substrate (6). In addition, Mueller et al. (35) detected the synthesis of acyl-type PAF (13.0–25.7%) when polymorphonuclear leucocytes were incubated with [^{3}H]acetate in the presence of various stimuli. Therefore, acetyltransferase may contribute to the formation of acyl-type PAF in the cells. These results suggest that the same acetyltransferase was responsible for the transfer of [^{3}H]-acetate from [^{3}H]acetyl-CoA to lyso-PAF or LPC, respectively. Therefore, the availability of lyso-PAF and LPC in vivo may exert a regulatory influence on the production of PAF. An interesting problem for future study is whether the acyl-type PAF has a different role from PAF.

Recently, Lee et al. (36) demonstrated that microsomal rat spleen preparations can synthesize alkyl-acetyl-glycerol by an acetyl-CoA:alkyl-lyso-*sn*-glycero-3-phosphate acetyltransferase and that this intermediate is subsequently dephosphorylated by an alkyl-acetyl-*sn*-glycero-3-phosphate phosphohydrolase to generate alkyl-acetyl-glycerol. Furthermore, they found that the acetyltransferase differs from the acetyltransferase responsible for the transfer of acetate from acetyl-CoA to lyso-PAF. The biosynthesis of these unique diglyceride analogs that possess a short chain acyl group at the *sn*-2 position could have important ramifications other than just in the formation of PAF, since in some systems oleoyl-acetyl-glycerols (37) and alkyl-acetyl-glycerols (38) can exert potent biological effects, such as the activation of protein kinase C and the induction of cellular differentiation, respectively. However, no definitive conclusion can be reached until the enzymes are purified and the properties studied.[1]

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[1]After submission of this manuscript, a paper on the partial purification and characterization of 1-O-alkyl-2-lyso-*sn*-glycero-3-phosphocholine:acetyl-CoA acetyltransferase from rat spleen was published (Gomez-Cambronero, J., Velasco, S., Sanchez-Crespo, M., Vivanco, F., and Mato, J.M. (1986) *Biochem. J. 237* 439–445); the activity in the presence of LPC was about 12% of that observed with lyso-PAF.

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Two New Icosapentaenoic Acids from the Temperate Red Seaweed *Ptilota filicina* J. Agardh

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Two new fatty acid metabolites, 5(Z),7(E),9(E),14(Z),17(Z)-icosapentaenoic acid and 5(E),7(E),9(E),14(Z),17(Z)-icosapentaenoic acid, have been isolated from the temperate red marine alga, *Ptilota filicina* (Ceramiales, Rhodophyta). The structures of these new compounds, isolated as their methyl ester derivatives, have been deduced from detailed ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR and 2D-NMR analyses as well as comparisons to known compounds.
Lipids 22, 190–194 (1987).

The widespread occurrence of ω3 fatty acids in marine organisms is a unique feature of marine-derived lipids with considerable health and economic consequences (1–6). Further, relatively simple biochemical modifications of arachidonic and icosapentaenoic acids results in molecules possessing important hormonal and bioregulatory functions in mammalian systems (7–9). Hence, we have been interested in examining metabolites of fatty acid origin from Oregon coastal seaweeds as part of our evaluation of the biomedicinal potential of these marine plants. The lipid extract of *Ptilota filicina* was identified in our survey efforts as strongly antimicrobial to gram-positive and -negative bacteria and possessed several unique secondary metabolites by thin layer chromatographic (TLC) analysis. A series of subsequent large-scale recollections have yielded, following extensive purification work, several new and uniquely functionalized 20 carbon fatty acids. The structures of two of these—5(Z),7(E),9(E),14(Z),17(Z)-icosapentaenoic acid (1) and 5(E),7(E),9(E),14(Z),17(Z)-icosapentaenoic acid (3)—are reported here (Scheme 1).

Other species of the genus *Ptilota* from elsewhere in the world have been examined previously for new biomedicinal agents. From *P. pectinata* collected from Norway, the amino acid taurine was identified (10); from *P. plumosa* from the coast of Great Britain, a potent hemagglutinin activity with human B cells has been described (11–13). *P. filicina* from the Soviet Union has been examined as a possible food source due to reported high concentrations of essential amino acids (14). However, Oregon coastal seaweeds have not been previously examined in detail, presumably due to the combination of adverse collection conditions and fewer reports of unique terpenoid natural products from temperate zone algae. Hence, the natural products of *P. filicina* from Oregon were unstudied prior to this work.

1 R = H
2 R = CH_3

3 R = OH
4 R = OCH_3
5 R = N(pyrrolidine ring)

SCHEME 1

EXPERIMENTAL METHODS

Ultraviolet spectra were recorded on an Aminco DW-2a UV-Vis spectrophotometer and infrared spectra (IR) on a Perkin-Elmer 727 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on Varian EM 360, FT-80A and Bruker AM 400 NMR spectrometers, and all shifts are reported relative to an internal TMS standard. Low resolution mass spectra (LRMS) were obtained on a Varian MAT CH7 spectrometer, while high resolution mass spectra (HRMS) were obtained on a Kratos MS 50 TC. High performance liquid chromatography (HPLC) was done using a Waters M-6000 pump, U6K injector and R 401 differential refractometer, while thin layer chromatograms were made using Merck aluminum-backed TLC sheets (silica gel 60 F_{254}). All solvents were distilled from glass prior to use.

P. filicina was collected from exposed intertidal pools (−0.5 to +0.5 m) at Marine Gardens on the Oregon coast in June 1985. Voucher specimens are on deposit at the Department of Botany Herbarium at Oregon State University. The seaweed was preserved by freezing until workup, at which time the defrosted alga (1.522 kg dry weight) was homogenized in warm $CHCl_3$/MeOH (2:1, v/v). The mixture was filtered and the solvents were removed in vacuo to yield a residue that was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ was dried over $MgSO_4$, filtered and reduced in vacuo to yield 17.3 g of a dark green tar. The crude extract was fractionated by silica gel chromatography in the vacuum mode (10 cm × 9 cm, Merck TLC-grade Kieselgel), and metabolites were

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progressively eluted with increasingly polar mixtures of isooctane and EtOAc. Those eluting with 25–45% EtOAc/isooctane yielded a mixture of fatty acids containing compounds 1 and 3. Treatment of a portion of these fractions with CH_2N_2 afforded a mixture of methyl esters (696 mg), which was subsequently chromatographed on a gravity-driven silica gel column (2.5 cm × 55 cm, Woelm Kieselgel 70–230 mesh) using isocratic conditions (EtOAc/isooctane, 1:9, v/v). This yielded a simplified mixture of 2 and 4 (296 mg) from which each could be isolated by normal phase HPLC (Alltech Si Gel column, 25 cm × 10 mm, 2.0% EtOAc/isooctane) to give 71.5 mg of 2 and 44.3 mg of 4, both as colorless oils.

Methyl 5(Z),7(E),9(E),14(Z),17(Z)-icosapentaenoate (2). Compound 2 was a colorless mobile oil showing the following: UV (MeOH) λ_{max} 253, 262, 273 nm (ε = 56,880; 74,570; 56,370); IR ($CHCl_3$) ν 3010, 2925-2875, 1735, 1450, 1000, 920 cm^{-1}. For ^1H NMR and ^{13}C NMR data, see Table 1.

Methyl 5(E),7(E),9(E),14(Z),17(Z)-icosapentaenoate (4). Compound 4 was also isolated as a colorless mobile oil and showed UV (MeOH) λ_{max} 258, 268, 279 (ε = 45,000; 57,000; 44,000); IR ($CHCl_3$) ν 3015, 2930-2870, 1735, 1440, 1000 cm^{-1}. For ^1H NMR and ^{13}C NMR data, see Table 1.

N-Icosa-5(E),7(E),9(E),14(Z),17(Z)-pentaenoyl pyrrolidine (5). The pyrrolidide derivative 5 was obtained via the following literature procedure (15): At 0 C, freshly distilled pyrrolidine (1 ml) was added to a partially pure sample of 4 (12.2 mg), glacial acetic acid (0.1 ml) was added and the solution was maintained at 20 C. After 24 hr, the reaction was terminated by quenching with water, and the products were repetitively extracted with $CHCl_3$ (3 × 25 ml). The $CHCl_3$ layer was first washed with 1 M HCl (3 × 25 ml) and then with H_2O (3 × 25 ml); it then was evaporated in vacuo to obtain 9.2 mg of crude product. Final purification of compound 5 (ca. 1.8 mg) was achieved using HPLC (Waters μ-Porasil 8 mm × 50 cm, 40% EtOAc in isooctane) and showed the following: ^1H NMR (bz-d-6) δ0.91 (3H, t, J=7.7 Hz), 1.21 (4H, m, N-CH_2*CH_2*), 1.39 (2H, p, J=7.4 Hz), 1.93 (4H, m), 2.03 (4H, q, J=7.2 Hz), 2.16 (2H, q, J=6.9 Hz), 2.65 (2H, t, J=6.5 Hz, N-*CH_2*), 2.79 (2H, bt, J=6.3 Hz), 3.38 (2H, t, J=6.4, N-*CH_2*), 5.45 (4H, m), 5.60 (2H, m), 6.18 (4H, m). Low resolution electron impact mass spectrometry (LR EIMS) m/z (rel. intensity): 356 (M^+)+H (1.7), 355 (M^+) (6.7), 340 (0.2), 326 (0.3), 312 (0.4), 300 (0.3), 286 (1.8), 272 (0.5), 260 (0.3), 246 (1.4), 232 (1.0), 218 (1.1), 2.04 (0.6), 192 (0.6), 178 (0.6), 166 (1.4), 152 (2.4), 140 (1.8), 126 (5.9), 113 (100), 98 (19.3), 70 (26.2), 55 (46.1); HR EIMS m/z, obs. 356.2969 (M^+)+H, $C_{24}H_{38}NO$ requires 356.2955.

RESULTS AND DISCUSSION

The red seaweed *P. filicina* (Ceramiales) grows abundantly in the mid-intertidal zone along the central Oregon coast (16). Based on the results of our survey for biomedicinals from Oregon seaweeds, a large recollection was made in June 1985 and maintained frozen until extracted for its lipids using standard methodology. Conventional silica gel vacuum chromatography of this dark green oily tar gave several fractions containing a brown charring, UV active compound. By infrared (IR) and ^{13}C NMR analyses, these fractions were a mixture of carboxylic acids that were rendered separable following derivation to the corresponding methyl esters (CH_2N_2). Final purification of these derivatives was achieved using silica gel column chromatography followed by HPLC and yielded two unstable colorless oils (2 and 4), the structures of which were deduced from spectroscopic data as outlined below.

It was recognized early in the structure elucidation process that 2 and 4 were geometrical isomers of one another, due to the similarity in spectroscopic properties of the two molecules and the spontaneous room temperature conversion of 2 and 4, monitored by HPLC. Further, the characteristic ultraviolet absorptions for a *c,t,t* triene functionality (λ_{max} = 253, 262, 273) in 2 were replaced in the absorption spectra of 4 with those characteristic for a *t,t,t*-triene (λ_{max} = 258, 268, 279) (17). Hence, the greater instability of 2 was explained by its propensity to double bond isomerization as well as autoxidation and presumed polymerization (18–20).

As neither 2 nor 4 gave meaningful mass spectral information (electron impact, chemical ionization), the more stable metabolite derivative, 4, was converted into the corresponding pyrrolidide (5) (21). Derivative 5 was characterized by LR EIMS (obs. M^+ 355 [6.7%]) and HR EIMS (obs. [M^+]+H) to yield a molecular formula of $C_{24}H_{37}NO$. Hence, the corresponding molecular formula

TABLE 1

NMR Data for the Methyl Ester Derivatives of Two Icosapentaenoic Acid Natural Products from *P. filicina*[a]

	Compound 2				Compound 4			
	^1H			^{13}C[b]	^1H			^{13}C[c]
C No.	δ	m	J(Hz)	δ	δ	m	J(Hz)	δ
1	—	—	—	174.01	—	—	—	174.01
2	2.33	t	7.5	33.36	2.31	t	7.4	33.35
3	1.73	p	7.5	24.82	1.73	p	7.4	24.51
4	2.23	q	7.5	27.08	2.09	m	—	32.08
5	5.33	m	—	130.78[d]	5.57	dt	14.4,7.2	132.64[d]
6	6.04	bt	11.4	129.76[d]	6.08	m	—	131.34[d]
7	6.35	dd	13.9,11.4	125.75[d]	6.08	m	—	130.66[d]
8	6.15	m	—	130.78[d]	6.08	m	—	131.46[d]
9	6.10	m	—	130.15[d]	6.08	m	—	130.57[d]
10	5.71	dt	14.4,7.2	135.07[d]	5.63	dt	14.4,7.2	134.35[d]
11	2.09	m	—	32.37	2.09	m	—	32.34
12	1.46	p	7.5	29.21	1.46	p	7.4	29.25
13	2.09	m	—	26.69	2.09	m	—	26.68
14	5.33	m	—	129.59	5.33	m	—	129.62
15	5.33	m	—	128.46	5.33	m	—	128.49
16	2.77	bt	6.1	25.56	2.77	bt	6.0	25.56
17	5.33	m	—	127.30	5.33	m	—	127.31
18	5.33	m	—	131.82	5.33	m	—	131.86
19	2.09	m	—	20.55	2.09	m	—	20.55
20	0.97	t	7.6	14.29	0.97	t	7.6	14.29
1′	3.66	s	—	51.49	3.66	s	—	51.48

[a]Chemical shift values in ppm relative to TMS as an internal standard operating at 9.398 T. All spectra obtained in $CDCl_3$.

[b]Assignments by comparison with values determined for 4 and with several model compounds (24,25,27).

[c]Assignments from a ^1H-^{13}C heteronuclear 2D shift correlation spectroscopy experiment and by comparisons with model compounds (24,25,27).

[d]Carbons assigned by comparison to model *c,t,t* and *t,t,t* conjugated trienes (27).

for both natural products (1 and 3) was $C_{20}H_{30}O_2$, yielding six degrees of unsaturation.

Further structure elucidation efforts were conducted with derivative 2, principally because most of its protons were clearly resolved in its high field ^{1}H NMR spectrum (Table 1). The ^{13}C NMR spectrum of 2 showed one ester carbonyl, confirmed by a characteristic stretch at 1735 cm^{-1} in the IR, and 10 olefinic carbon atoms, thus accounting for all six degrees of unsaturation. Other than these olefin and ester carbons, the ^{13}C NMR spectrum was composed of one methyl ester carbon, one aliphatic methyl carbon and eight methylene carbons. Hence, 2 was deduced to be a methyl ester derivative of an icosapentaenoic acid containing a conjugated *c,t,t* triene as well as two nonconjugated double bonds.

The linear array of these methylene and olefinic groups in 2 was conveniently given by a ^{1}H-^{1}H 2D shift correlation spectroscopy (COSY) experiment (Fig. 1) (22). The C-2 protons, identified by their characteristic chemical shift (δ2.33) and triplet multiplicity (23), were correlated to a 2H signal at δ1.73 (H_2-3), which was further correlated to another 2H signal appearing at δ2.23 (H_2-4). The allylic nature of these latter protons was indicated by both their chemical shift and a clear correlation to an olefin proton at δ5.33. As this latter signal overlapped four other protons at this chemical shift, observation of the continuity of this linearly related spin system was afforded by detection of allylic coupling between H_2-4 and H-6 (δ6.04). The H-6 proton was correlated both to the H-5 proton at δ5.33 and the H-7 proton at δ6.35. The H-7 signal was, in turn, correlated to H-8 (δ6.15), H-8 to H-9 (δ6.10) and H-9 to H-10 (δ5.71). Coupling constant analysis (Table 1) of this C-5 to C-10 olefin constellation reconfirmed the *c,t,t* nature of the triene, as well as fixing its orientation relative to the carboxyl group.

A correlation between H-10 and two protons of a 6H multiplet at δ2.09 identified the allylic protons at C-11. This latter multiplet was coupled in the upfield region only to the terminal methyl group and a 2H signal at δ1.46. This latter signal must, therefore, be H_2-12. In turn, these C-12 protons were coupled exclusively to the 6H multiplet at δ2.09, and therefore, H-13 must also be allylic. Further, since the 6H multiplet was only coupled in the olefin region to one proton at δ5.71 (H-10) and to two of five overlapping protons at δ5.33, H-14 must be located in this latter multiplet.

At the other end, the terminal methyl group (δ0.97) was correlated to the 6H multiplet of overlapping allylic protons at δ2.09 and thus must contain, in addition to H_2-11 and H_2-13, H_2-19. In analogy to the reasoning used to

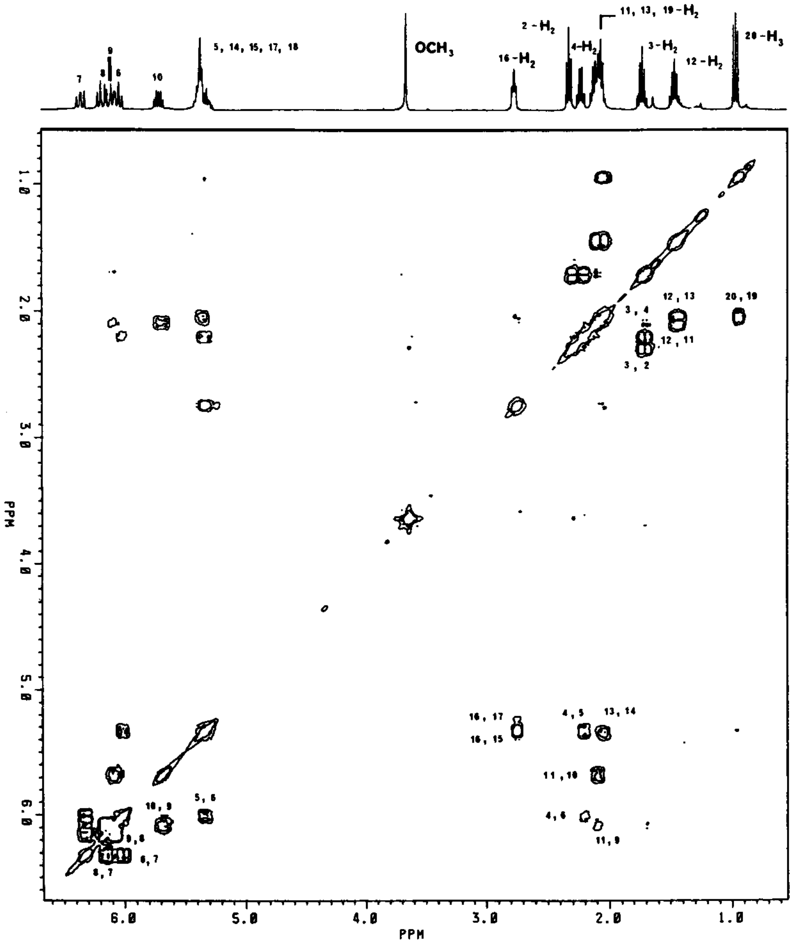

FIG. 1. ^{1}H-^{1}H correlation spectroscopy (22) of methyl 5(Z),7(E),9(E),14(Z),17(Z)-icosapentaenoate (2) showing correlations between coupled protons (ca. 7 mg of 2 in 0.4 ml $CDCl_3$ with 0.3% TMS, 5-mm tube, 400 MHz).

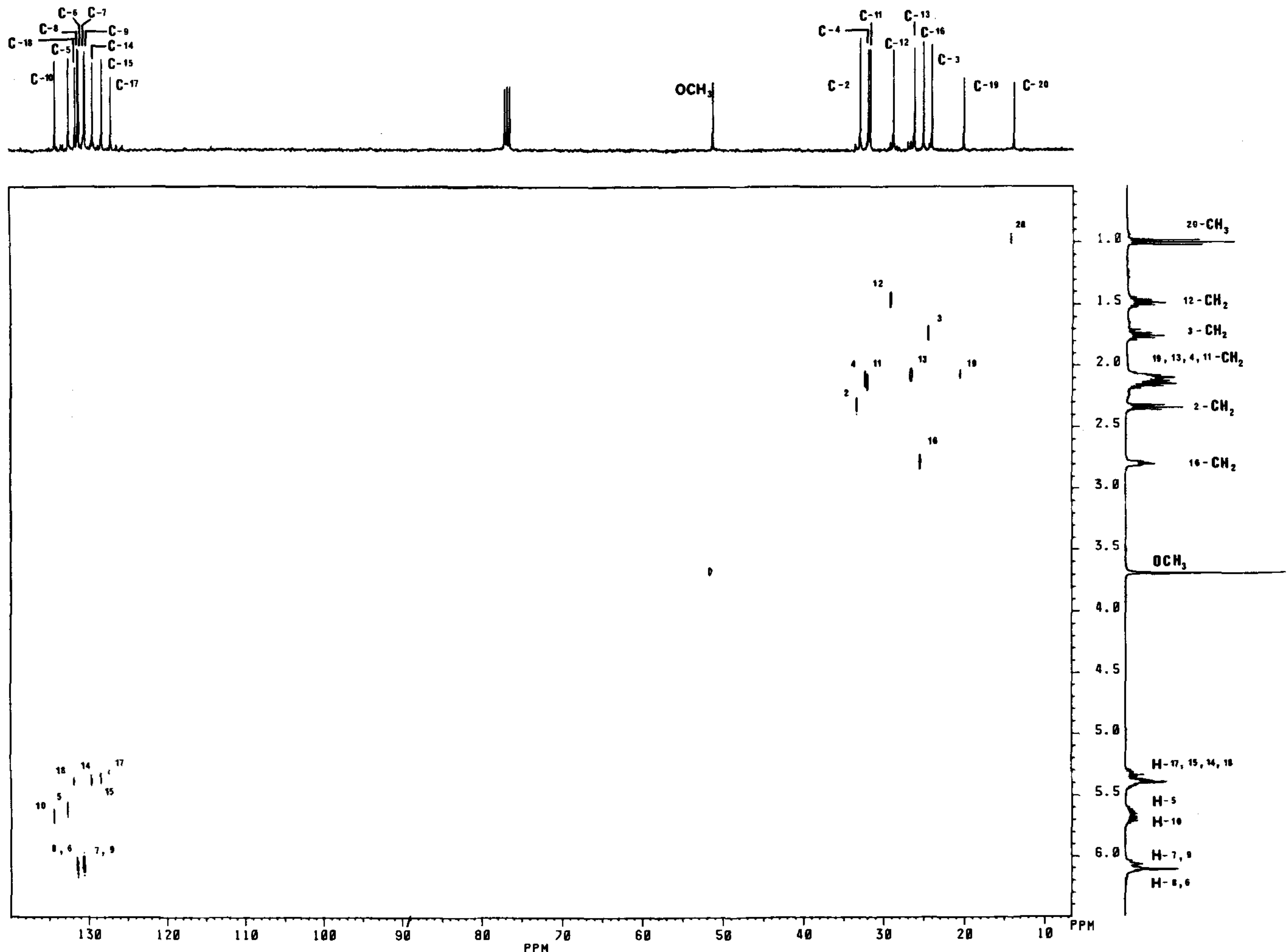

FIG. 2. ^1H-^{13}C heteronuclear 2D shift correlation spectroscopy (22) of methyl 5(E),7(E),9(E),14(Z),17(Z)-icosapentaenoate (4) showing one bond coupling (^1J) correlations between carbons and their respective protons (ca. 20 mg of 4 in 0.4 ml $CDCl_3$ with 0.3% TMS, 5-mm tube; ^1H NMR at 400 MHz, ^{13}C NMR at 100 MHz).

identify the H-14 chemical shift, the H-18 proton must also be in the 5H multiplet at δ5.33.

A third partial structure for derivative 2 was identified beginning with a bisallylic methylene, identified as such by its characteristic chemical shift (δ2.77) and multiplicity (t, J=6.1 Hz) (23). In the COSY experiment, these protons were exclusively correlated to two of the five olefin protons occurring as an multiplet at δ5.33. By a ^1H-^{13}C heteronuclear 2D shift correlation spectroscopy experiment (Fig. 2) (22), the carbon shift of the bisallylic methylene was identified as δ25.56; hence, via comparison with all four geometrical isomers of the model compound, methyl-12,15-octadecadienoate, both olefins were of the Z geometry (24).

Consideration of these three partial structures with regards to the molecular formula and number of olefins in 2 required two of the olefins to be duplications. With two connections to be made between these three partial structures, and with the middle fragment being symmetrical, only one structure, 2, was possible.

The structure of derivative 4 was developed by comparison of its spectroscopic properties with those of derivative 2 (Table 1). In most respects, the major structural features present in 2 were also present in 4, as was suggested by the facile conversion of 2 into 4. The major difference was that the *c,t,t* triene in 2 was replaced in 4 by a *t,t,t* triene. This was evidenced by UV (see experimental), ^1H NMR ($J_{5\text{-}6}$ = 14.4 Hz, $J_{9\text{-}10}$ = 14.4 Hz) and ^{13}C NMR data (6 olefin carbon signals greater than 130 ppm) (25).

It is likely that both natural products 1 and 3 are derived by isomerization of the $C_{8\text{-}9}$ and $C_{11\text{-}12}$ olefins in 5(Z),8(Z),11(Z),14(Z),17(Z)-icosapentaenoic acid to the $C_{7\text{-}8}$ and $C_{9\text{-}10}$ positions. Metabolites 1 and 3 were shown to be true natural products of *P. filicina* by nearly identical TLCs for the lipid extracts obtained by standard extraction methodology and by a procedure described to inhibit extraction artifacts resulting from enzyme-catalyzed degradation of complex lipids (26).

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METHODS

Analysis of Volatile Fatty Acids in Biological Specimens by Capillary Column Gas Chromatography

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A method was developed to analyze and quantify volatile fatty acids (VFA) such as acetic, propionic, butyric, isobutyric, valeric and isovaleric acids in biological specimens. To obtain good sample transfer into the chromatographic system an organic solvent had to be used together with an aqueous milieu, thus improving wetting properties of the liquid sample plug introduced into the column. Sample preparation was carried out under alkaline conditions in order to exclude or minimize sample losses due to sample transfer during the extraction and work-up procedure. A cold on-column injection was applied to avoid irregular discrimination of the various acids due to sample splitting and an automatic injection technique was used to accommodate the large number of samples generated from biological origin. Connection of a pre-column of wide internal diameter (0.53 mm) to the analytical column (0.32 m) was optimized and adapted to the nature of the injection solvent mixture consisting of acetonitrile, water and hydrochloric acid. To obtain well-separated and correctly quantifiable gas chromatographic peaks, it was essential to perform the chromatography under acidic aqueous conditions. Standard resolution conditions and response factors were evaluated. The chromatographic results of applying this method to biological specimens from both rats and humans are provided.
Lipids 22, 195–200 (1987).

Volatile fatty acids (VFA) include components such as acetate, propionate, butyrate, isobutyrate, valerate and isovalerate. These components are described as being volatile since they can be steam distilled under acidic conditions, but they are also classified as short chain fatty acids due to their low molecular weights. Some of these compounds are produced during the normal metabolic processes, while all of them are produced in the large intestines of mammals, including humans, via fermentation pathways. It is this latter route of production that includes our research interest. VFA are produced when dietary components such as fibers, α-galactosides and unabsorbed starch and sugars are fermented. Recent evidence indicates that there are several potentially beneficial physiological effects of VFA production and absorption. These include their abilities to serve as an energy source (1,2) and their influence on the differentiation and growth of colonic epithelial cells (3,4). To study these effects, it is necessary to have a rapid, sensitive and accurate method for analyzing these constituents in biological specimens such as intestinal tissues, plasma and saliva.

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Separation, identification and quantification of these volatile fatty acids have most commonly been done using gas liquid chromatography. However, the stationary phase of columns must be sufficiently polar to separate the acids without exhibiting excessive retention of the constituents, and it must also be efficient at low temperatures so that pyrolysis and decomposition of the constituents or of the stationary phases do not occur (5). A primary difficulty with this method of analysis, in the case of packed columns, has been the interaction between the acids and the column packing, which results in tailing of peaks (reversible adsorption) and retention of the acids on the column (irreversible adsorption). With subsequent injections, these constituents may be released, and this phenomenon has been referred to as "ghosting" or "memory effect." This type of absorption has been reduced by using glass columns in preference to stainless steel columns (5), by incorporating phosphoric acid into the packing materials (5) and by incorporating formic acid into the carrier gas stream (6). To some extent, these problems have been overcome by using capillary column chromatography (6). Recently, these constituents were reported to be separable by employing a fused silica capillary column coated with OV-351, which is a Carbowax nitroterephthalic acid polymer, and a split injection mode chromatography procedure (6). A similar technique has been used by others to analyze these constituents in human urine (7). Other workers (8) reported quantifying both volatile and nonvolatile organic acids as the t-butyldimethylsilyl derivatives following capillary chromatography using OV-17, a coating material of intermediate polarity.

A cold, on-column mode injection system and an immobilized phase composed of polar coating material were used in developing an alternate procedure for separating and quantifying VFA using capillary column chromatography. With this procedure, accurate and reproducible analyses of VFA were performed in extracts prepared from biological samples including plasma, saliva, intestinal contents and intestinal tissues.

MATERIALS AND METHODS

Materials. Routine separations of the acids were made using a standard mixture containing acetic, n-propionic, isopropionic, n-butyric, isobutyric, n-valeric and isovaleric acids (WFA-2, Supelco, Gland, Switzerland). Isocaproic acid (Fluka AG, Switzerland) was added to this mixture and served as an internal standard for purposes of quantification. Acetonitrile (Romil Chem., Shepshed, England; HPLC grade) was used without further purification, and it was necessary to use a high-purity grade solvent so that there was no measurable contamination with acetic acid. Hydrochloric acid, methanol, acetone, hexane (all from Merck, Darmstadt, Federal Republic of Germany),

tridecane (Fluka) and sodium chloride (Merck) were used as supplied. Water was deionized and distilled.

Apparatus. A fused-silica capillary column (25 m × 0.32 mm ID) was coated with Carbowax 20M (Applied Science Labs., State College, Pennsylvania) using a procedure fully described elsewhere (9), and a desired film thickness was 0.30 μm. A fused-silica (1 m × 0.53 mm ID) precolumn was also utilized after applying a procedure in which it was washed, using suction, with a solution of 5% (w/v) γ-glycidoxypropyltrimethoxysilane (Union Carbide, New York, New York) in methanol. The Carlo Erba Mega HRGC 5160 chromatograph used was equipped with an automatic liquid on-column injector (Carlo Erba AS-550). Data were generated and acquired using a Spectra-Physics 4700 integration system.

In general, the chromatography was conducted using hydrogen as carrier gas at a flow of 2.5 ml/min and a pressure of 0.6 kPa. The precolumn was connected to the analytical column by means of a purged-butt connector that facilitated entrance of hydrogen as a make-up or auxiliary gas to flush the union, and the analytical column was inserted into the precolumn by at least 1 mm. The flame ionization detector was operated at 300 C with gas flows of 300 ml/min for air and 30 ml/min for hydrogen. Nitrogen (ca. 20 ml/min) was used as make-up gas. The injector block was air-cooled and was equipped with a secondary cooling system. The injection was made directly into the column at the point that it entered the oven.

Use of a precolumn was found to be necessary to extend the lifetime of the analytical column. This fused silica precolumn was of 0.53 mm id and was treated prior to being installed with a silane monomer (9) followed by washing with ca. 10 ml of the acidic/aqueous/organic solvent mixture. Furthermore, we were able to intermittently remove the precolumn from the chromatograph, and to flush the precolumn with this solvent mixture so that accumulated impurities and salts could be removed. This precolumn and its treatment significantly extended the lifetime of the analytical column.

Sample preparation and assay procedure. Standard curves were derived by the addition of analytes and internal standard (isocaproic acid) to the solution composed of the desired solvent:water/HCl ratio. The calibration ranges were from 0–800 nmol/ml for each acid. The ratios of the integrated areas for the seven VFA were calculated as a percentage of the integrated areas for 400 nmol of internal standard, and these ratios were plotted against concentration of the analyte to generate the standard curves. Linear regression analysis was then used to determine the quantity of VFA in a sample to which had been added 400 nmol of internal standard. The extractions of VFA from biological specimens were performed using the alcoholic extraction procedure of Remesy and Demigne (5), except that KOH was used to alkalinize the extracts in place of NaOH, and the ratio of ethanol:water was increased from 5:1 to 8:1.

RESULTS

Fused silica columns coated with Carbowax 20M to desired film thicknesses ranging from 0.20–0.50 μm were prepared. Those columns with film thicknesses of 0.20–0.30 μm were found to give the best separations of the six VFA and the internal standard (isocaproic acid), and the peak shapes tended to be sharper, which allowed more accurate quantification. In our experience, better resolution of these seven components was achieved with immobilized Carbowax 20M as a coating material than with OV-351.

The choice of solvent had a significant effect on the general appearance of the solvent front and baseline as well as the resolution of the compounds. Six solvents—acetone, acetonitrile, hexane, methanol, tridecane and water—were evaluated. None provided acceptable results when used in a single solvent system. Solvent peaks tended to be broad for methanol and acetone, and this interfered with elution of the components of interest. However, when a solvent/water mixture was used, the characteristics of the solvent peak were tremendously improved.

Solvent. Water mixtures of 8:2 were generally found to produce the most desirable results. Of the six solvent systems evaluated, acetone/water and acetonitrile/water were found to be the most favorable, since they provided excellent characteristics in showing a rapid return to baseline following the elution of the solvent, and the seven compounds were fully resolved. By contrast, resolution of the components was poor when methanol/water was used as solvent; the immiscibility of some of these solvents with water precluded their use. When water was used as the sole solvent, the peak shapes of the acids were generally poor.

The degree of acidification had a marked effect on the chromatography of the acids. Without adequate acidification, the compounds failed to elute as distinct, quantifiable peaks. Additionally, ghosting, or the appearance of components from one injection during subsequent injections, and retention time shifts were serious problems. These problems were overcome by adjusting the acidity so that the peaks were discrete and unchanged by further addition of acid. In general, we found that using 1 N HCl in the presence of 80% of the other organic solvent provided excellent results. Nonetheless, it was necessary to add additional HCl if samples were stored for more than 18 hr prior to their analyses. It should be emphasized, however, that there were no observable undesirable consequences of "overacidifying" since no further changes were detected in peak shape or area ratios of the acids to internal standard once the critical, but minimum, quantity of acid had been added.

Temperature programming was used in all analyses. We found that the characteristics of the solvent peaks were best when initial temperatures of 60–75 C were used. At higher starting temperatures, the solvent peak was excessively large. Following a time lag of 2 min at the initial temperature, a constant increase at 8 C/min was found to provide optimum resolution of the components. Under these conditions, the compound of highest molecular weight, isocaproic acid, was eluted within 10–12 min. Nonetheless, continuous high quality resolution was only achieved if the oven temperature was cycled to 180–200 C and held at that temperature for at least 5 min.

A typical chromatogram showing the separation of the six VFA and isocaproic acid as internal standard is provided in Figure 1. The seven components are distinctly separable. Reproducibility of duplicate injections were

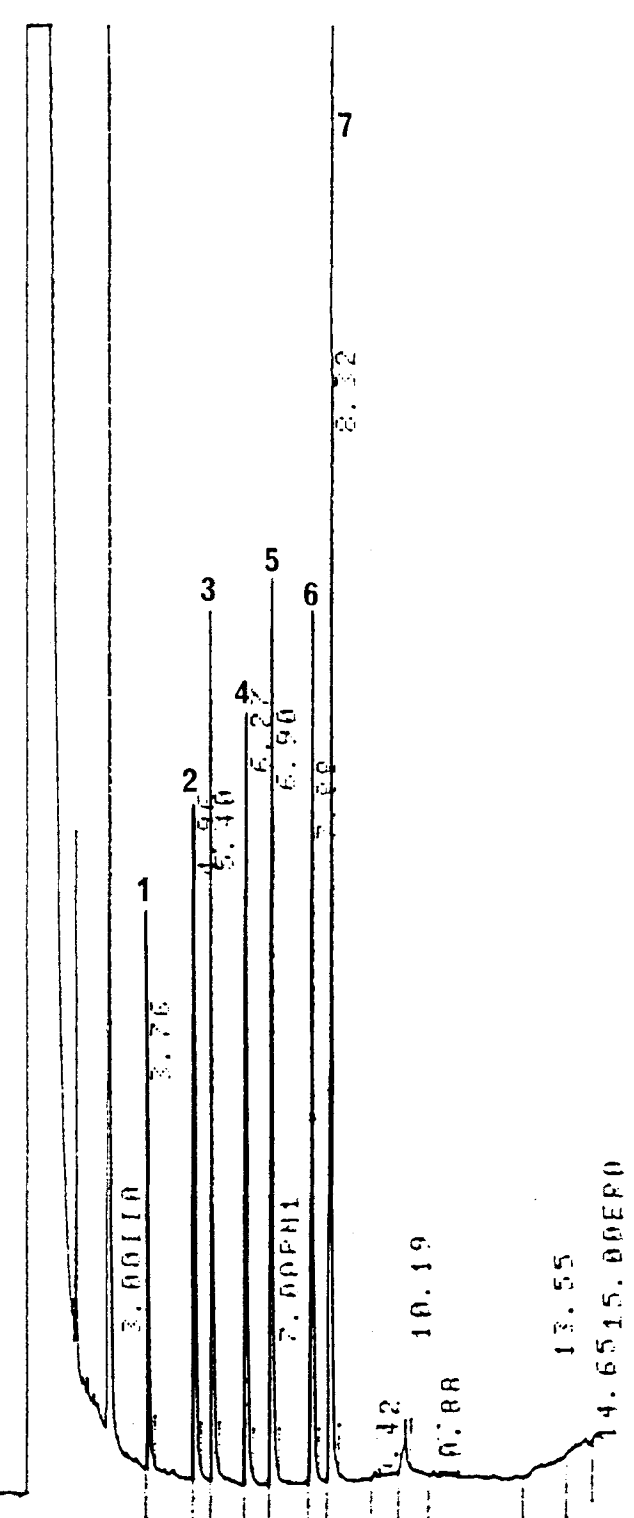

FIG. 1. Chromatogram of standard VFA (WSFA-2, Supelco Inc.) with isocaproic acid as internal standard. Peak identifications: 1, acetic acid; 2, propionic acid; 3, isobutyric acid; 4, n-butyric acid; 5, isovaleric acid; 6, n-valeric acid; 7, isocaproic acid. Solvent: acetonitrile/H_2O; temp. prog: 65–145 C; injector: cold, on-column; detector: FID, 300 C.

found to be within 5%, when the area ratios of the VFA:internal standard were calculated. Additionally, the response was found to be linear between the range of at least 0–800 nmol/ml when a 1 μl injection was made (Fig. 2), and this linearity may have continued although higher concentrations were not evaluated. We estimate that the lower limits of detection were in the range of 20 nmol/ml and that the lower limits for quantification were in the range of 50 nmol/ml. The identity of the component that corresponded to the sharp peak that eluted before peak 1 (Fig. 1) was not determined but, since the purity of the WSFA-2 solution was not indicated, it is likely that this peak could have been formic acid.

The reproducibility with which we could analyze VFA in biological specimens closely approximated the reproducibility of our standards. In particular, the coefficients of variation of replicate injections for standard VFA, which had not undergone any of the routine extraction procedures necessary as part of the analytical procedure used to quantify VFA in biological specimens, ranged from 2–5%. When these same standards were subjected to the alcoholic extraction procedure, the coefficients of variation were unchanged. When biological specimens were similarly extracted and repeat injections made, the coefficients of variation ranged from 3–6% for specimens including plasma, cecal contents and cecal tissue of rats, and plasma and saliva from humans. Typical chromatograms of extracts prepared from these biological specimens are shown in Figure 3. It is worth noting that in

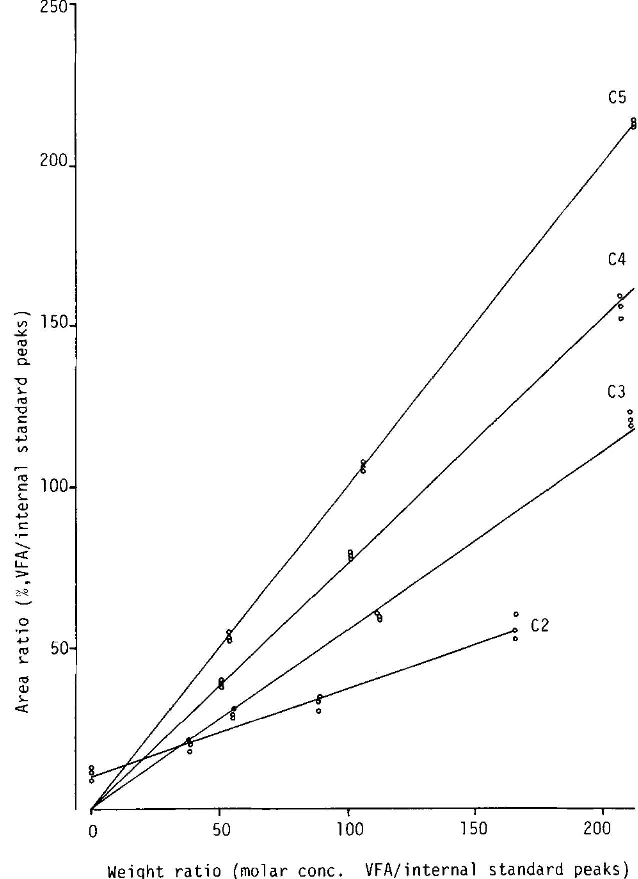

FIG. 2. Area ratio of VFA: internal standard peaks vs weight ratio of VFA: internal standard. Values were determined at 4 weight ratios for each of 4 standard VFA. Triplicate analyses were made on three days using newly prepared solutions.

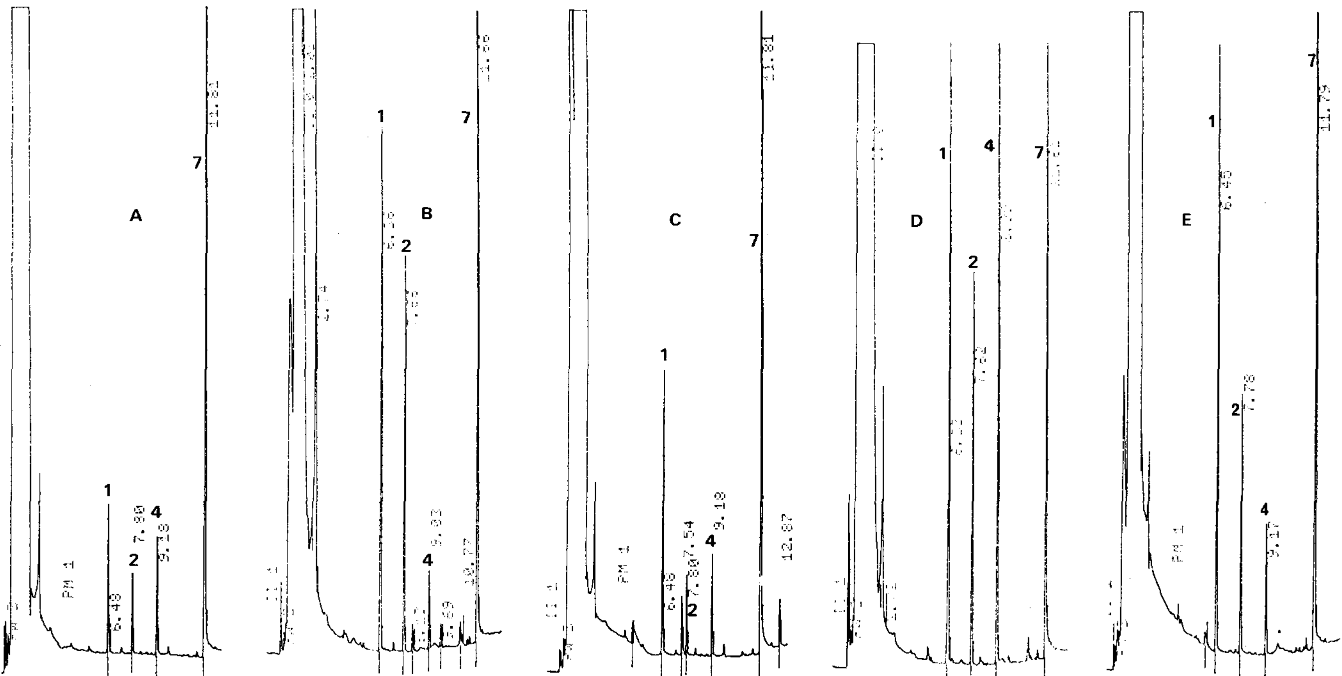

FIG. 3. Chromatograms of VFA in alcoholic extractions of biological specimens including human plasma, 300 mg (A); human saliva, 100 mg (B); rat portal plasma, 200 μl (C); rat cecal contents, 100 μl, 1:10 dilution (D); and rat cecal tissue, 100 μ, 1:10 dilution (E). Each chromatogram is shown in the presence of ca. 400 nmol of isocaproic acid as internal standard.

some of these biological specimens some higher molecular weight constituents were observed, but these did not interfere with further chromatographic analyses.

DISCUSSION

The objective of this research was to develop a capillary chromatography system to analyze volatile fatty acids in large numbers of biological specimens. Thus, we needed to have readily quantifiable chromatograms and columns that would remain functional during many analyses. Additionally, it was necessary to be able to perform each analysis quickly and with a minimum of preparation. To meet these objectives, we wished to avoid derivatization. This necessitated performing the chromatography in a solvent matrix consisting of water/hydrochloric acid together with an organic solvent.

Although other workers have reported using a polar phase such as OV-351 to separate the volatile fatty acids and their isomeric constituents (6), we found that these constituents were better resolved on a stationary phase of the polyethylene glycol type. Previously, we have reported using immobilized Carbowax 20M columns for the analyses of such lipids as methyl esters of long chain fatty acids and prostanoids (10–12). To prepare these columns, we used a technique in which silane monomers are grafted onto the polymer chains followed by cross-linking between the grafted units, which effected phase immobilization as previously reported (9). This polymer was found to be sufficiently stable to withstand repeated loads of the acidic/aqueous/organic solvent mixture used in the solubilization of underivatized volatile fatty acids, and it could also withstand large sample volumes of up to 10 μl without phase stripping effects. Additionally, this phase proved to be efficient and durable, since we were able to make more than 1000 analyses on a single column over a period of ca. 3 months using an automatic injection mode.

Due to the volatile nature of these acids, consideration was given to the nature of the injection system. An on-column injection mode was thought to be desirable since it avoids the discrimination of constituents, which may occur with the split-injection technique and a septum purge. Additionally, the on-column injection mode reduces the potential for degradation of the highly polar compounds, which may occur in an acidic medium if a flash evaporation technique is used such as occurs in a heated injector.

Given the desirability of using a direct injection system and a precolumn, it was necessary to use a precolumn that had a larger diameter (0.53 mm) than that of the analytical column (0.32) to accommodate the injection needle (ca. 0.4 mm). The precolumn had another positive side effect. Creating, by definition, a retention gap (13,14), this helped to avoid possible peak splitting, which otherwise could have occurred. The two columns were connected via a purged butt connector, using a technique that avoided dead volumes and backflush. In addition to the usual connection mode, a restricting valve can be installed in this gas line for precisely adjusting the auxiliary gas flow relative to the precolumn length. The conformation of the system is diagrammatically presented in Figure 4. The degree to which the pressure is restricted by this valve distinctly influences gas chromatographic performance, and this can most easily be observed by monitoring the elution shape of the solvent, as is shown by the examples of an incorrectly and a correctly purged butt connector (Fig. 5). Efficient flushing of this connector is also

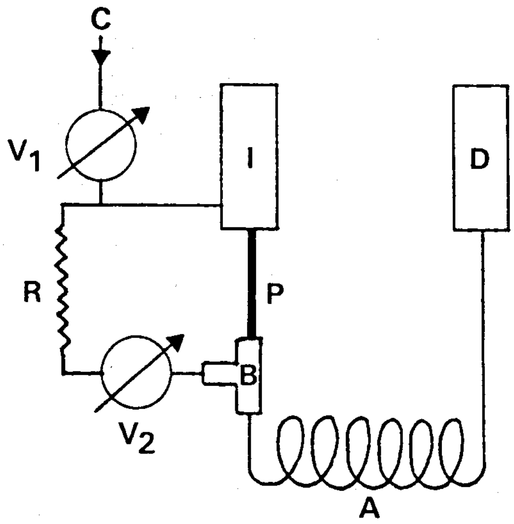

FIG. 4. Physical conformation of the analytical column, precolumn and carrier gas streams. A, analytical column; B, butt connector; C, carrier gas inlet; D, detector; I, injector; P, precolumn; R, restriction; V_1, carrier gas valve; V_2, fine metering valve.

essential in order to avoid the deposition or accumulation of noneluted and partially degraded compounds on the column. This would lead to rapid deterioration of column performance.

The optimum initial oven temperature for resolution of the VFA contained within this solvent system was found to be between 60-75 C. At these temperatures, peak resolutions were excellent, acetic acid was clearly separated from the solvent peak system and the overall speed of analysis was acceptable. At higher initial temperatures of ~80 C, an excessively large solvent peak was observed, and this interfered with the quantification of VFA; at lower initial temperatures of ~50 C, the speed of analyses was unnecessarily slow. The initial temperature was maintained for 1-2 min to allow elution of the solvent peaks, and this was followed by temperature programming at 8 C/min to 140 C. At this temperature, all VFA were eluted, but the column was still found to contain impurities that could be eluted by more rapidly heating the column to 200 C and maintaining this temperature for at least 5 min. Thus, the column was "purged" prior to the next injection, and the impurities were not allowed to accumulate. Nonetheless, we found that impurities collected

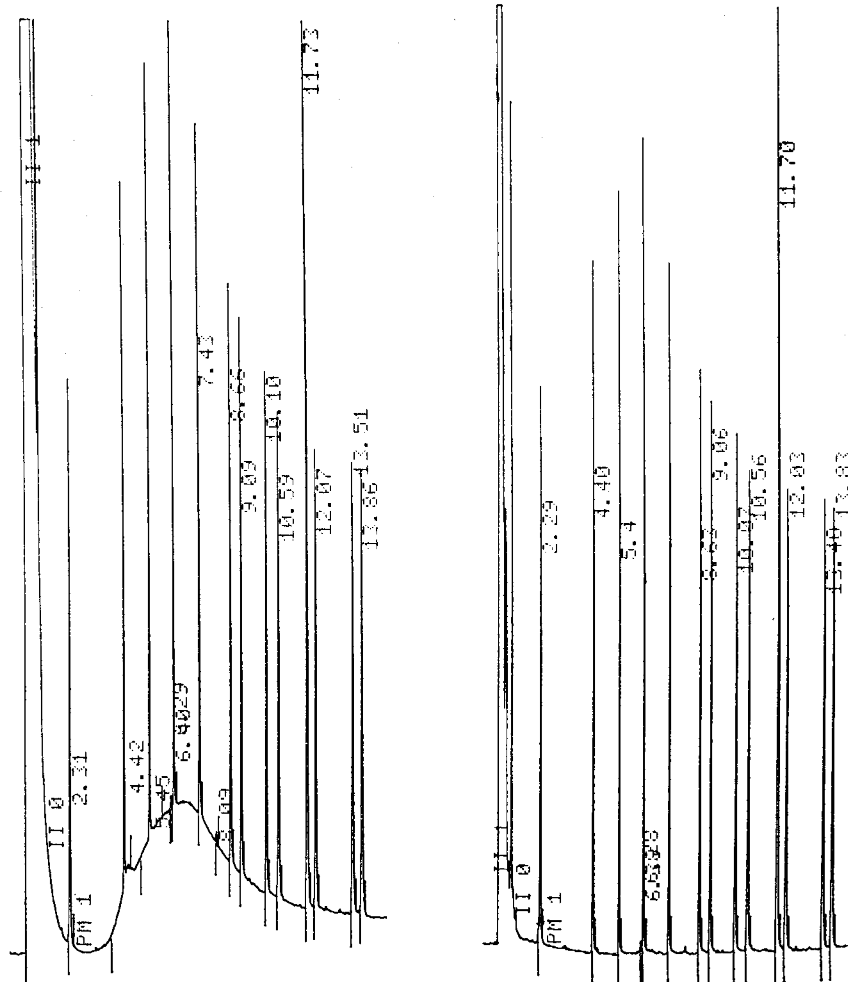

FIG. 5. Influence of proper purging of the butt connector on the elution shapes of the solvent peaks. Left chromatogram unpurged; right chromatogram purged. Peaks correspond to fatty acid methyl esters C8-C17.

in the precolumn over time, and this necessitated cleaning by cutting away an initial section or by rinsing with the injection solvent mix. These cleaning procedures were repeated roughly every 100 injections, and the total number of injections which could be made on one length of precolumn varied from 400–500. By caring in this way for the precolumn, we were able to make more than 1000 injections on a single analytical column. Thus, our experiences showed that the immobilized Carbowax columns were highly resistant against water, acid and solvent stripping.

Various solvent systems were evaluated, and the choice was based on extraction of VFA from biological specimens and consideration of the underivatized character of the acids. Thus it was necessary to avoid solvents that would favor dissociation of the acids that would cause peak broadening and reduce the accuracy of quantification. Water as the sole solvent was found to cause excessively broad peaks, and it appeared to cause the formation of an intermediate pseudo-stationary film that interacted in the chromatographic process. Thus, since water could not be used alone as solvent for the analyses of the underivatized acids, it was necessary to use solvents that could function as a co-solvent with this aqueous phase. Thus, water served as the primary solvent for the free acids, and the organic phase had primary importance in sample transfer and in determining chromatographic resolution. VFA would not disperse in hexane or ether, and the immiscibility of these solvents with the acidified aqueous phase precluded their use. When methanol was used as solvent, we observed a remarkably low response. Acetone or acetonitrile, in combination with an acidified aqueous phase, provided excellent resolution of the VFA, high responses and identical chromatographic performance. However, acetone could not be found in an absolutely acetic acid-free quality, and since the degree of contamination was not constant, quantification of acetic acid in biological specimens was impeded. Thus, acetic acid-free acetonitrile was taken as the preferred solvent. However, since acetic acid or a co-eluting component was found to be generated within this solvent system over long periods of time, it was necessary to simultaneously prepare and analyze calibration standards and samples so that this change could be eliminated from the calculated results. When this was done, the quantification of acetic acid in biological specimens was found to be constant over at least a 3-day storage period. Thus, the final composition of the injection sample consisted of acetonitrile, water and aqueous hydrochloric acid in proportions that could be variable within the limits previously described. Samples injected in such a medium gave sharp, quantifiable peaks (Fig. 3) with highly reproducible results.

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The Use of Iodine Staining for the Quantitative Analysis of Lipids Separated by Thin Layer Chromatography

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The method described for quantitative estimation of lipids separated on thin layer chromatography plates exploits the observation that most lipids can be stained by iodine vapor and that, in "controlled" conditions, the intensity of this staining is proportional to the actual amount of lipid in the spot. The method consists of i) exposing the developed plate to iodine vapor; ii) spraying it with a suitable solvent to prevent halogen evaporation; iii) collecting the stained lipids by scraping the spots off the plate; and iv) determining by a rate-sensing method the absorbed iodine. The final determination is performed by measuring spectrophotometrically (at 410 nm) the rate of decolorization of a solution of Ce(IV) by As(III) in strong acidic conditions. The reaction rate, which is positively related to the concentration of iodine, is derived from the slope of the absorbance change plotted vs time.

Providing that standards and samples are stained simultaneously, a quantitative estimation of lipid components of a mixture is possible in a reasonable time with excellent accuracy and reproducibility. In our hands, the method has been successfully applied to several common phospholipids, long chain fatty acids, cholesterol and deoxycholate, and triacylglycerols, in the range of 5–60 μg.
Lipids 22, 201–205 (1987).

Quantitation of lipid spots on thin layer chromatography (TLC) plates involves two steps: visualization of lipids and quantitative (chemical or densitometric) analysis.

One of the most widely used visualization procedures is the exposure of the developed plate to iodine vapor (1). Due to the hydrophobic character of both the halogen and lipids, samples adsorb iodine and readily appear on plates as brownish spots.

Early observations and our present experience (1,2) indicated that molecules as phospholipids, long chain fatty acids, triacylglycerols and cholesterol derivatives adsorb iodine proportionally to the amount of lipid material (up to 60 μg) on the TLC plate. This is true, however, only if iodine evaporation from the plate is immediately blocked and the exposure of plates is not exceedingly long. Under these conditions, a staining procedure widely used for qualitative purposes may become useful also for quantitative determinations.

It must be pointed out that the method described here allows a significant time savings, since it requires less than 30 min of effective work and uses common laboratory equipment and reagents.

This method has not been tailored for routine chromatographic measurements or for very specialized laboratories devoted to lipid chemistry. Rather, this procedure is proposed for investigators who might have occasional need of a rapid and suitable quantitative analysis of lipids.

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MATERIALS AND METHODS

Egg yolk sphingomyelin (containing primarily palmitic acid), cardiolipin (pig heart), phosphatidylinositol (containing primarily linoleic and palmitic acids), dipalmitoylphosphatidylserine, linoleic, oleic and elaidic acids, cholesterol and sodium deoxycholate were purchased from Sigma (St. Louis, Missouri); triolein (glycerol trioleate) was from BDH (Deventer, The Netherlands). All these substances were used without further purification and were solubilized or diluted in chloroform or chloroform/methanol (2:2, v/v) to give final concentrations (gravimetrically assessed) of 2.5 mg/ml.

Precoated silica gel 60 TLC plates with or without fluorescent indicator (0.2 mm thick) were obtained from Merck (Darmstadt, Federal Republic of Germany). The spotting solvent was chloroform or chloroform/methanol (2:1, v/v).

Equipment consisted of an Eppendorf microfuge, a chromatographic tank (22 × 10 × 22 cm) and a Beckman UV 5230 spectrophotometer with a built-in strip-chart recorder. Readings were taken at 410 nm. Normally, full scale was set at O.D. 2 and the chart speed at 2 inches/min.

For the arsenious acid reagent, sodium arsenite (Baker, Poole, England) was dissolved (with heating and stirring) in 0.15 M sulfuric acid to give a final concentration of 0.01 M. After cooling, the solution was divided into small aliquots and stored in the refrigerator. These solutions are stable for long periods. Before analysis the arsenite reagent was diluted 20-fold with 0.15 M sulfuric acid; this solution is hereafter referred to as solution A.

For the ceric-ammonium sulfate reagent, ceric sulfate and ammonium sulfate (Baker) were dissolved (with heating and stirring) together in 2.8 M sulfuric acid to give final concentrations of 0.05 and 0.15 M, respectively. The solution was centrifuged and the clear deep yellow supernatant decanted and divided into small aliquots. These solutions (referred to as solution B) are stable for several months at room temperature.

Commercial TLC plates are available with or without fluorescent indicators and may contain several other substances as binders or salts. Because it cannot be assumed that all these substances do not affect the reactions described below, plates, different in type and source, should not be considered necessarily interchangeable.

Preliminary experiments should be performed to assess the possibility of using the various types of commercially available plates. Most of our studies were performed on Merck Silica gel 60 plates (20 × 20 cm) with or without fluorescent indicator. However, a few experiments carried out with similar plates from different commercial sources gave essentially the same results.

Lipids were dissolved in suitable solvents (chloroform or chloroform/methanol). Amounts ranging from 5 to 60 μg were spotted on TLC plates with the aid of a small glass syringe with a Teflon tip fitted with a micrometric device (Radiometer SBU1A). Any device for precise and reproducible delivery of solutions on plates may be used.

Iodine crystals were placed in a round plastic dessiccator and allowed to vaporize at room temperature until the jar was saturated with iodine vapor. The plate was quickly put in the dessiccator horizontally with its four corners based on the inner round edge of the container. The gel side was toward the transparent cover of the jar to allow a rapid inspection of the staining.

Several durations of exposure of the plates to iodine vapors were tried, but 90 sec was found to be satisfactory for most of the tested substances. Some lipids (e.g. phosphatidylinositol, phosphatidylethanolamine), however, required a longer exposure (120 sec), especially at minimal concentrations of lipids. The lipids appear as brown spots on a white background (after longer exposure the background tends to become yellowish).

Densitometric measurements were performed using a Shimadzu CS 920 densitometer.

The principle of the assay method has been discussed extensively in a previous paper (3). In brief, the assay is based on the ability of iodine to act as catalyst in the ceric arsenite reaction (4). Since the formation of the reaction product (CeIII) results in a dramatic decrease in the absorption of the solution at 410 nm, the kinetics of this process may be easily measured by monitoring the rate of decolorization. The change in absorption at 410 nm follows zero order kinetics and may be conveniently monitored spectrophotometrically (the use of a strip-chart recorder is advisable). The initial velocity (initial reaction rate) is proportional to iodine concentration, which is, in turn, proportional to the amount of lipid in the spot. The lipid concentration in an unknown sample may be easily estimated by comparison with a standard curve prepared simultaneously with the same material. In a typical assay, a mixture of lipids (the amount of each component should not exceed 60 μg) is spotted on the left corner (ca. 2 cm from the bottom) of a chromatographic plate and developed as needed. At the end of the run, the plate is removed from the tank and let to dry at room temperature. With the aid of a micrometric device, scalar volumes of standards are regularly placed on the large area of the plate that has not been used for the chromatographic separation. After drying, the plate is moved into a jar containing iodine crystals and stained by exposure (normally 90 sec) to iodine vapor (Fig. 1). The plate is removed and immediately sprayed with glacial acetic acid. All the round brown areas are marked with a glass tip, carefully scraped off the plate and transferred into plastic Eppendorf vials (1.5 ml). After addition of 1 ml of 1.8 M sulfuric acid, the samples are vigorously shaken on a vortex (1-2 min) and, finally, centrifuged on a microfuge (5 min). Blanks consisting of background areas (having approximately the size of the broader sample spot) are similarly treated. The supernatants (0.8 ml) are collected in small capped tubes, where they can be stored for 48 hr or more. In fact, iodine determinations performed at various times on the same samples gave essentially similar results after a few minutes to up to 60 hr (Fig. 2). To perform the final iodine quantitation, the samples (e.g., 50 μl) are diluted fivefold with 1 M sulfuric acid. This dilution diminishes the chances of accidental transfer of silica gel particles in the optical cuvette, thus preventing possible artifactual interferences. Furthermore, if the lipid amount in the sample is rather small, the dilution step may be skipped or proportionally reduced. Two-hundred-and-fifty μl of this diluted solution is transferred directly to a cuvette (plastic disposable cuvettes are convenient to use).

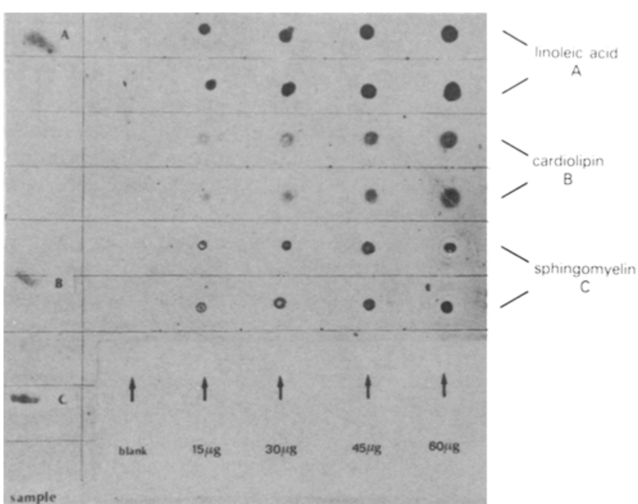

FIG. 1. An example of quantitative assay of a simple lipid mixture. Left side: linoleic acid, 25 μg (A, top), pig heart cardiolipin, 25 μg (B, middle) and egg yolk sphingomyelin (containing primarily palmitic acid), 30 μg (C, bottom), after their chromatographic separation on a silica gel 60 plate (chloroform/methanol/water [67:25:3, v/v/v]). Right side: Scalar amounts of each of the above substances. Arrows indicate the relative quantities. Standards have been loaded on the gel immediately after the sample separation and before the exposure to iodine vapor. By this procedure, staining of samples and standards is accomplished simultaneously under identical conditions.

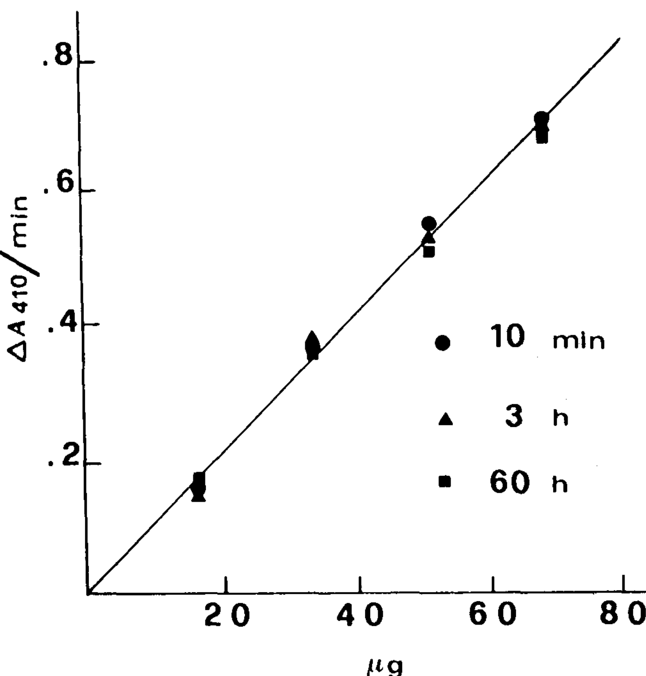

FIG. 2. Acetic acid prevents evaporation of iodine from stained plates. Quantitative determinations of dipalmitoyllecithin performed at various intervals of time (10 min-60 hr) after spraying the iodine-stained plate. Essentially the same reaction rate is observed in all determinations.

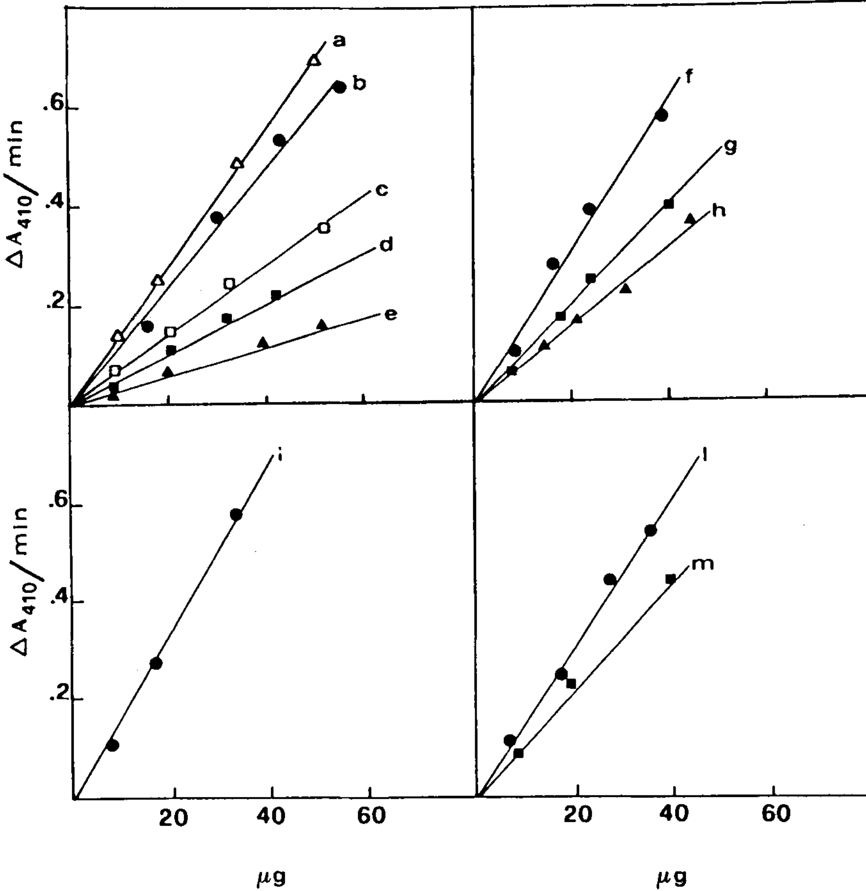

FIG. 3. Standard curves (in the range 5–60 μg) obtained with various lipids: a, egg yolk sphingomyelin (containing primarily palmitic acid); b, cardiolipin (from pig heart); c, phosphatidylinositol (containing primarily linoleic and palmitic acids); d, dipalmitoyllecithin; e, dipalmitoylphosphatidylserine; f, linoleic acid; g, oleic acid; h, elaidic acid; i, triolein (glycerol trioleate); l, sodium deoxycholate; m, cholesterol.

Six-hundred μl of solution A (arsenite) and 60 μl of solution B (ceric-ammonium sulfate) are then sequentially poured in the same cuvette. After rapid mixing by inversion, the cuvette is placed in the spectrophotometer, set at 410 nm for reading. The calibration curve is obtained by plotting the initial rate (i.e., the slope of the change in absorbance vs time) expressed as ΔA/min vs the lipid (iodine) content of standards. The actual amount of lipid in a mixture is readily obtained by reading the values off this curve, the amount corresponding to the measured rate (ΔA/min).

Figure 3 shows several standard curves obtained using different types of lipid molecules. It seems clear that the response is correlated to lipid saturation (linoleic acid > oleic acid > elaidic acid). Specific details are given in the figure legend.

Precision of the assay was evaluated as between-day or day-to-day precision using lipid extracts from three amniotic fluids containing increasing amounts of lecithin (palmitoyl lecithin was used as standard) for a period of 15 days (Table 1). The procedure for lipid extraction and separation was that of Gluck et al. (5).

TABLE 1

Precision of Assay

Sample	μg of lecithin		
	1	2	3
Precision between day	18.0 ± 1.2	31.1 ± 1.8	46.2 ± 1.7
Precision day to day	17.2 ± 1.4	31.5 ± 2.0	46.9 ± 2.4

Precision has been assessed over a period of 15 days. Samples 1, 2 and 3 are three amniotic fluids containing three different levels of lecithin. The extraction of lecithin and its separation has been performed according to Gluck et al. (5).

The same three amniotic fluids used for determining precision were used for accuracy. It was assessed by comparing our procedure with a well accepted method (6). In this case, the ratio of lecithin to sphingomyelin (μg/μg) was densitometrically measured according to the original

TABLE 2

Accuracy

Sample	1	2	3
R_1 (μg/μg)	1.2	2.3	2.8
R_2 (μg/μg)	1.4	2.5	2.7

R_1 indicates the ratio of lecithin to sphingomyelin in three amniotic fluids determined by means of our method; R_2 is the same ratio densitometrically determined according to Gluck et al. (6).

paper (5) and compared with the ratio obtained by making use of our method (Table 2).

DISCUSSION

A variety of methods for the quantitative estimation of lipids separated by TLC are today available. The lipids are usually visualized and subsequently quantitated by either charring or staining, followed by either direct densitometric (or planimetric) measurement or by scraping the spots off the plate for chemical wet analysis.

Charring simply involves spraying the TLC plate with sulfuric acid or with sulfuric acid containing a strong oxidizing agent, followed by heating at high temperature for various times. Although this method appears to be generally suitable, nevertheless it is known that staining by charring is sensitive to lipid saturation, is influenced by the silica gel and is dependent on the time and temperature of heating. Moreover, when present, the oxidizing agent may affect the result because of the possible loss of carbon by conversion to CO_2.

Dye staining, a nondestructive method, usually allows recovery of material from the plates, so that scraped spots may be used for further analysis. Different types of staining have been proposed over the years. Some have general application (2,7-10), while others make use of more specific reagents and are used when the lipid molecules contain phosphorus, choline, sialic acid or other groups of particular reactivity (11-16). Even in these instances, however, there are frequent problems with background discoloration or fading of spot colors, especially when the spots have to be read by densitometry or directly evaluated by planimetry.

Various other problems have been described after spraying plates with ammonium sulfate, bismuth subnitrates (6,17), etc.

Another widely applied method of staining lipid molecules on TLC chromatographic plates is the exposure of the plate to iodine vapor in a sealed tank. This method is extremely simple, sufficiently sensitive and almost universally applicable. Also in this case, however, staining is dependent on the nature of the lipid molecule (i.e., some molecules stain more than others) and is dependent on the time of exposure to the halogen vapor. Furthermore, once the plate is removed from the container, the brown spots begin to fade within a few minutes and more or less rapidly tend to disappear.

The method presented in this paper makes use of iodine staining by introducing a simple procedure to hinder the evaporation of the halogen. Under controlled conditions and within a relatively wide range of concentrations, it appears that the amount of trapped iodine is linearly related to the amount of adsorbing lipid. The subsequent estimation of iodine is performed by exploiting a rate-sensing method developed in our laboratory a few years ago (3): by this technique we have successfully applied the procedure to various lipid molecules, by making use of appropriate calibration curves, prepared from standards placed on the same plate where the chromatographic separation has taken place.

The method has proved to be largely reliable and reproducible with several test substances, providing that evaporation of iodine from the plates is effectively hindered. After several attempts, we have found that uniform spraying with acetic acid, a solvent in which iodine is highly soluble, is the most effective treatment for this purpose.

The observation that various lipid molecules, even in the same quantity, stain quite differently, does not represent a serious problem. To circumvent this obstacle, in fact, it is possible to reduce up to fivefold the preliminary dilution (see Methods) of the sample before the final spectrophotometric measurement. The assay is performed in a reasonable time, is not particularly cumbersome, does not require special attention or skills and, most important, is highly reproducible. Another important advantage comes from the fact that the assay is a rate-sensing method: the superior accuracy of true kinetic methods over others is generally recognized and, in regard to iodine determination, has been extensively discussed in a previous paper (3).

It must be emphasized that since the response (the change in the absorption of cerium) is correlated with the lipid saturation (see standard curves of Fig. 3), the quantitation of unknown samples is impossible unless the same material is available for calibration curves. However, the linearity of the response observed with synthetic and natural mixtures of lipids indicates the possibility of using such mixtures as standards of a total lipid in tissue or biological fluid extracts, providing that the approximate lipid composition is known.

Bearing in mind such restrictions, the present method may be conveniently used when the occasional need of lipid analysis does not justify the acquisition of dedicated optical devices, such as densitometers, and when turnaround time may be critical for the immediate prosecution of the research work.

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Measurement of Lipid Peroxidation In Vivo: A Comparison of Different Procedures

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A study was undertaken to investigate whether some of the methods commonly used to detect lipid peroxidation of cellular membranes in vivo correlate with each other. The study was performed with the livers of bromobenzene-intoxicated mice, in which lipid peroxidation develops when the depletion of glutathione (GSH) reaches a threshold value. The methods tested and compared were the following: i) measurement of the malondialdehyde (MDA) content of the liver; ii) detection of diene conjugation absorption in liver phospholipids; iii) measurement of the loss of polyunsaturated fatty acids in liver phospholipids; and iv) determination of carbonyl functions formed in acyl residues of membrane phospholipids as a result of the peroxidative breakdown of phospholipid fatty acids. Correlations among the values obtained with these methods showed high statistical significances, indicating that the procedures measure lipid peroxidation in vivo with comparable reliability. Analogously, the four methods appeared also to correlate when applied to in vitro microsomal lipid peroxidation.
Lipids 22, 206–211 (1987).

During the past 30 years, a great deal of experimental evidence has accumulated suggesting that lipid peroxidation in cellular membranes is implicated in a variety of pathological conditions. These include liver cell injury by a number of toxins (CCl_4 and other halogenated hydrocarbons [1–6]; the effect of bromobenzene and other aryl halides acting as glutathione [GSH] depleting agents [7–9], injury due to other hepatotoxins, probably including ethanol [10]); lung damage after exposure to nitrogen dioxide (11) and ozone (12) or intoxication with the herbicide paraquat (13); increased red blood cell permeability and hemolysis associated with vitamin E deficiency (14); retrolental fibroplasia (15); paroxysmal nocturnal hemoglobinuria (16); abetalipoproteinemia (17); cell damage caused by ionizing radiation (18); and several aspects of oxygen toxicity (19).

Because of the growing importance of lipid peroxidation in the biomedical field, an increasing need for reliable methods to detect these processes has been perceived. Many methods are available to measure the extent of lipid peroxidation, but most are based upon the measurement of the products originating from the process at different stages. These methods include (20) i) the classic thiobarbituric acid (TBA) reaction to measure malondialdehyde (MDA) (21); ii) detection of the UV absorption characteristic of conjugated dienes (22); iii) fluorescent analysis of lipid peroxidation products (23); iv) measurement of ethane and pentane formation (24,25); v) detection of chemiluminescence (26); vi) measurement of oxygen uptake (27); vii) measurement of the loss of polyunsaturated fatty acids in membrane phospholipids (28); viii) the detection of lipid hydroperoxides (29); and ix) measurement of specific aldehydes such as alkenals (30).

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The detection of lipid peroxidation in biological systems in vitro is relatively simple since, when the reaction has been blocked, it is conceivable that the measurement of a certain product in the sample represents a reliable estimation. On the other hand, when detection of lipid peroxidation in vivo is attempted, many problems arise: the oxidation product may be rapidly metabolized or removed from the tissue under study; it may have interacted with different cellular substances so that it cannot be found in the free form; or it may be formed as an artifact during sampling of the tissue and other technical procedures. For these reasons, the detection of lipid peroxidation in vivo has often puzzled the biochemical pathologist who is primarily concerned with understanding the in vivo events. The present work was undertaken to determine whether the methods more commonly used in various laboratories to detect lipid peroxidation in vivo correlate and to test the reliability of each individual method. The study was performed with the livers of bromobenzene-intoxicated mice, in which lipid peroxidation develops when the depletion of GSH reaches a threshold value (9). Bromobenzene hepatotoxicity represents a good model for the study of in vivo lipid peroxidation, since in this experimental condition the level of detectable lipid peroxidation is far greater than in the case of CCl_4 or $BrCCl_3$ hepatotoxicity (9,31).

The following methods were compared: i) measurement of the MDA content of the liver; ii) detection of diene conjugation absorption in liver phospholipids; iii) measurement of the loss of polyunsaturated fatty acids in liver phospholipids; and iv) measurement of the carbonyl functions formed in the acyl residues of membrane phospholipids as a result of the peroxidative breakdown of phospholipid fatty acids. The latter method was recently developed in our laboratory (32).

MATERIALS AND METHODS

Male NMRI albino mice (Ivanovas GmbH, Federal Republic of Germany) weighing 20–30 g and maintained on a pellet diet (Altromin-Rieper, Bolzano, Italy) were used.

In vivo experiments. The animals were fed a liquid glucose (20%) diet for two days before intoxication, according to the protocol of Wendel et al. (33), to decrease the hepatic GSH content. This regimen decreased hepatic GSH by about 50% as compared to laboratory chow-fed animals and increased the frequency of occurrence of lipid peroxidation in liver phospholipids.

Bromobenzene (C. Erba, Milano, Italy) mixed with two volumes of mineral oil was administered intragastrically under light ether anesthesia, at a dose of 15 mmol/kg body weight. Control mice received mineral oil alone. All animals were fasted after intoxication.

Eighteen hr after intoxication, the animals were killed by exsanguination under ether anesthesia, and the livers were quickly removed, rinsed in ice-cold saline, weighed, and divided into two portions. The first portion was

extracted with 24 vol of chloroform/methanol (2:1, v/v) according to the method of Folch et al. (34), and the extract was used for i) detection of diene conjugation absorption in liver phospholipids; ii) determination of the amount of carbonyl functions in liver phospholipids; and iii) determination of the fatty acid pattern of phospholipids.

The diene conjugation absorption of liver phospholipids was measured as described by Casini and Farber (35), after precipitation of the phospholipids with acetone according to Borgström (36). Typical UV spectra and the difference spectrum (intoxicated − control animal) are reported in Figures 1A and 1B, respectively.

The amount of carbonyl functions originating from the peroxidative breakdown of unsaturated fatty acids in cellular phospholipids was measured according to Benedetti et al. (32). Briefly, carbonyl functions were detected after derivatization with 2,4-dinitrophenylhydrazine (DNPH) and separation of phospholipids from neutral lipids and unreacted DNPH. The absorption spectrum of DNPH derivatives of liver phospholipids from the intoxicated animals is similar to that obtained with a mixture of dinitrophenylhydrazones of standard aldehydes (Fig. 1D, dashed line). Figure 1C shows the typical absorption spectra of DNPH-treated liver phospholipids from control and bromobenzene-treated animals 18 hr after poisoning. From the difference spectrum (intoxicated − control animals) obtained (see Fig. 1D, solid line), the content of carbonyl functions in liver phospholipids can be estimated using an average molar extinction coefficient of 25500 (37–39).

The decrease of polyunsaturated fatty acids (arachidonic and docosahexaenoic acids) was measured by determining the whole fatty acid pattern of acetone-precipitated liver phospholipids (36). Fatty acid methyl esters were prepared and purified as reported by Benedetti et al. (40). They were analyzed by gas liquid chromatography in a Fractovap apparatus Model GI (C. Erba) using a spiral glass column (2 m × 2 mm internal diameter) packed with 20% diethyleneglycol succinate on Chromosorb W. Other conditions were as reported previously by Benedetti et al. (41).

The second portion of each liver was used for determination of the tissue content of MDA, which was measured as follows: tissue samples were homogenized in ice-cold trichloroacetic acid (TCA) (1 g tissue + 1 ml 10%, w/v, TCA, plus 8 ml 5%, w/v, TCA, or equivalent amounts) in an Ultra Turrax homogenizer. After centrifugation, a volume of the supernatant was added to an equal volume of 0.6%, w/v, TBA, and the mixture was heated to 100 C for 10 min. The absorption spectrum was then recorded over the range of 480–600 nm. As can be seen in Figure 1, the spectra obtained (Figs. 1E and 1F, solid lines) were quite similar to that obtained with a MDA standard produced by acid hydrolysis of 1,1,3,3-tetraethoxypropane and run under the same conditions. The MDA concentration was calculated from the absorption at 532 nm (absorption maximum) of the difference spectrum (intoxicated − control animals) using a molar extinction coefficient of 1.56×10^5, as reported by Jordan and Schenkman (28); this was also recalculated from our own standards.

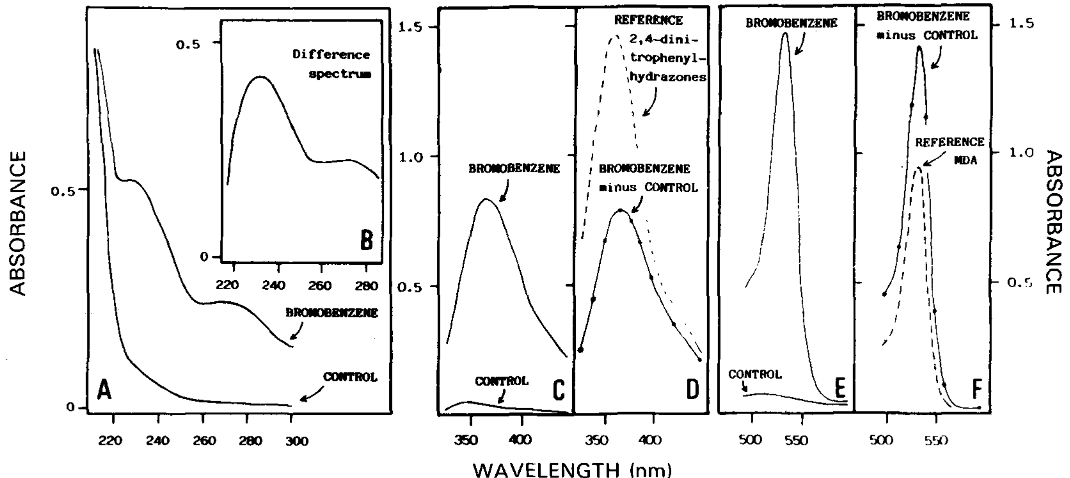

FIG. 1. A: Diene conjugation absorption in liver phospholipids derived from a control and a bromobenzene-poisoned mouse. The mice were killed 18 hr after intoxication (15 mmol/kg body weight, by mouth). Phospholipid concentration = 0.5 mg/ml in cyclohexane. B: Difference spectrum calculated from the spectra of panel A by subtracting the spectrum of the control sample from the one of the sample of the intoxicated animal. C: Absorption spectra of DNPH-treated liver phospholipids derived from a control and a bromobenzene-poisoned mouse (same animals as in A). The phospholipid concentration of the sample in chloroform was 0.5 mg/ml. The spectra of DNPH-treated samples were recorded against the corresponding blank samples in the reference cuvette. The blank samples contained the same phospholipid concentration, but were not treated with DNPH. See text and Benedetti et al. (32) for additional explanations. D: Solid line, difference spectrum calculated from the spectra of panel C by subtracting the spectrum of the control sample from the spectrum of the sample of the intoxicated animal. Dashed line, absorption spectrum obtained with a mixture of 2,4-dinitrophenylhydrazones of various aldehydes (4-hydroxynonenal, hexadecanal and decyl aldehyde, 20 nmol/ml for each aldehyde). E: Absorption spectra of the chromogen derived from the reaction of TBA with the TCA extracts of the livers derived from a control and a bromobenzene-poisoned mouse (same animals as in A). See text for additional explanations. F: Solid line, difference spectrum calculated from the spectra of panel E by subtracting the spectrum of the control sample from the spectrum of the sample of the intoxicated animal. Dashed line, absorption spectrum of the reaction product of TBA with standard MDA in TCA (6 nmol/ml).

The same TCA homogenate was also used for the determination of the tissue content of GSH according to Sedlak and Lindsay (42).

In vitro experiments. Mice were fasted overnight before use. The preparation of the microsomal fraction and the incubation of the microsomes were carried out as described by Benedetti et al. (32). The concentration of $FeSO_4$ in the incubation mixture was 60 μM. At the end of each incubation time, aliquots were drawn and divided into two portions: one was used for detection of the MDA formed as previously described (43); the other was extracted with 24 vol of chloroform/methanol (2:1, v/v) according to the method of Folch et al. (34). The lipid extract was then used for the detection of diene conjugation absorption, for the measurement of carbonyl functions in phospholipids and for the determination of the fatty acid pattern.

Other procedures. Protein content was determined according to the method of Lowry et al. (44). Serum glutamate-pyruvate transaminase (SGPT) activity was determined by an optimized UV enzymatic method (C. Erba).

RESULTS AND DISCUSSION

Table 1 shows the hepatic GSH depletion, liver necrosis (as assessed by the SGPT levels) and lipid peroxidation (measured by the hepatic MDA content, the diene conjugation absorption, the amount of carbonyl functions and the loss of polyunsaturated fatty acids in liver phospholipids) in the bromobenzene-intoxicated animals used in the present study. As was noted in previous studies (9), a large variation was observed in the sensitivity of individual animals to bromobenzene. There was, however, a readily evident relationship between the different parameters of the response to bromobenzene when the values for the individual animals were graphed together. As shown in Figure 2, a good correlation ($P < 0.001$) was found between the log of SGPT and the log of carbonyl functions, indicating that in each intoxicated animal lipid peroxidation is associated strictly with liver necrosis. The fact that 18 hr after the intoxication some animals showed high levels of lipid peroxidation while others revealed only minor values gave us the opportunity to study the relationships among the different methods for detecting lipid peroxidation in vivo. Figure 3 shows the correlations between (A) the hepatic content of MDA and the amount of carbonyl functions in liver phospholipids; (B) the hepatic content of MDA and the absorption of conjugated dienes in liver phospholipids; (C) the amount of carbonyl functions and the absorption of conjugated dienes in liver phospholipids; and (D) the hepatic content of MDA and the loss of polyunsaturated fatty acids (arachidonic and docosahexaenoic acids). All correlations showed high statistical significance, indicating that the methods

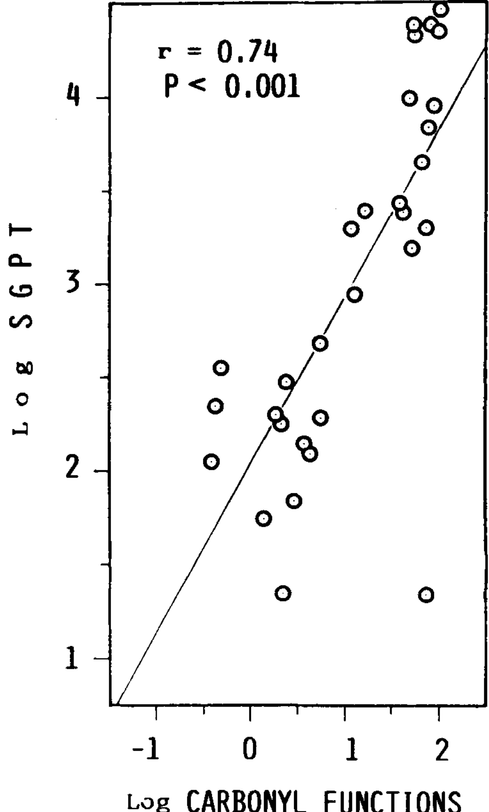

FIG. 2. Correlation between lipid peroxidation (as measured by the amount of carbonyl functions in liver phospholipids) and liver necrosis (as measured by SGPT levels). The parameters were expressed as log of the values obtained 18 hr after bromobenzene poisoning (15 mmol/kg body weight).

TABLE 1

Hepatic GSH Depletion, Liver Damage (SGPT Levels) and Lipid Peroxidation in Bromobenzene-Poisoned Mice[a]

	GSH (nmol/mg protein)	SGPT (U/l)	MDA (pmol/mg protein)	Diene conjugation absorption[b] ($\Delta A_{233\ nm}$)	Carbonyl functions (nmol/mg phospholipids)	PUFA (relative %) 20:4	PUFA (relative %) 22:6
Control	30.3±3.1 (8)	7±3 (6)	—	—	—	19.0±0.7 (7)	10.1±0.4 (7)
Bromobenzene-treated	1.8±0.2 (36)	1709±451 (36)	518.7±173.8 (23)	0.341±0.076 (30)	24.2±5.1 (43)	16.3±0.6[c] (32)	8.9±0.2[c] (32)

Values reported are means ± SEM. The number of animals is given in parentheses. Control values for MDA, diene conjugation absorption and carbonyl functions were subtracted from those of the intoxicated animals.

[a]MDA content of the liver, diene conjugation absorption, carbonyl functions and loss of polyunsaturated fatty acids (PUFA) in liver phospholipids.

[b]Phospholipid concentration: 1 mg/ml in cyclohexane.

[c]Significantly different from control, $P < 0.05$.

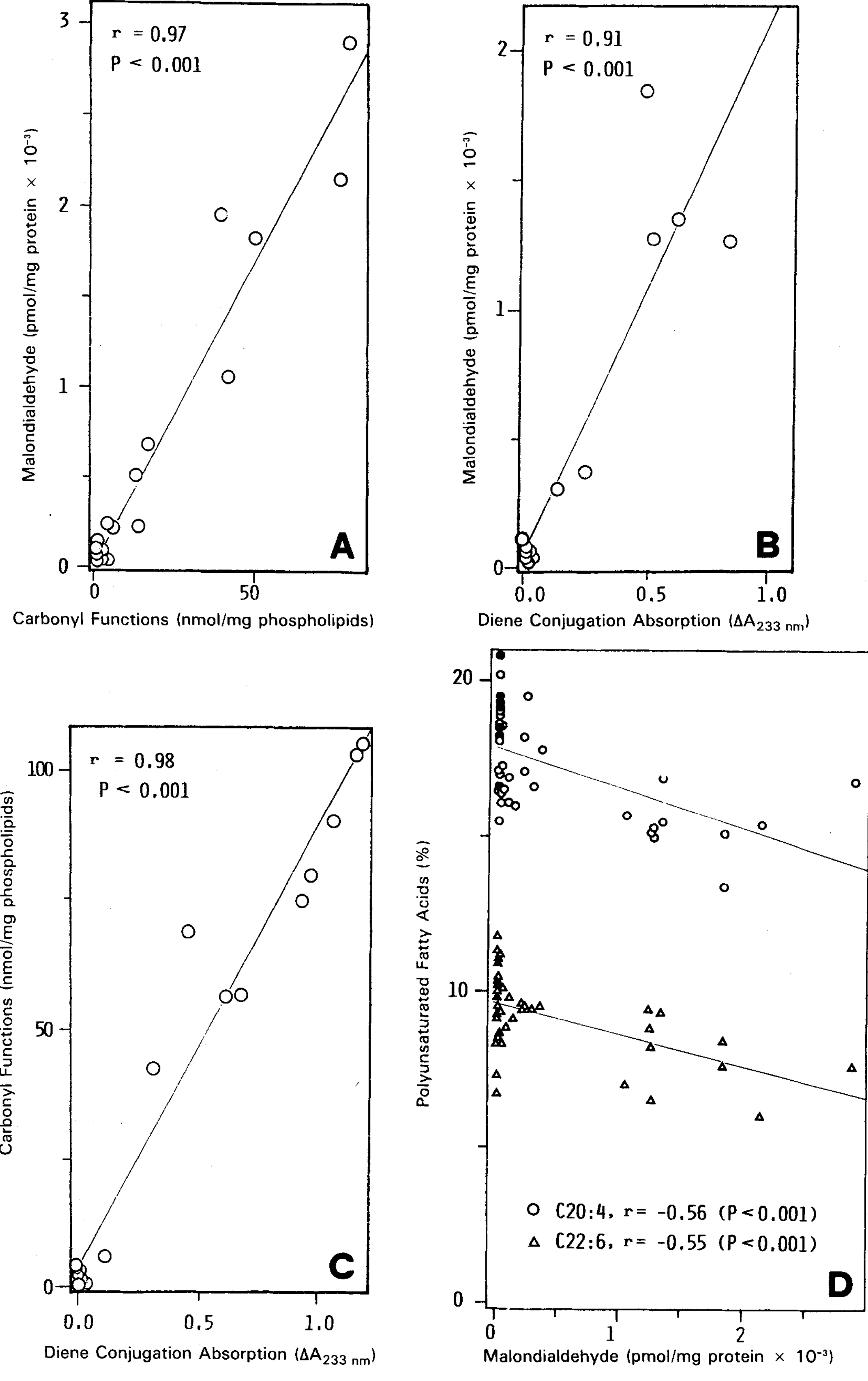

FIG. 3. In mice intoxicated with bromobenzene (15 mmol/kg body weight, by mouth), correlations between: A, hepatic content of MDA and carbonyl functions in liver phospholipids (PL); B, hepatic content of MDA and absorption of conjugated dienes in liver PL; C, carbonyl functions and absorption of conjugated dienes in liver PL. D, correlation between hepatic content of MDA and relative percentage of arachidonic (○) and docosahexaenoic (△) acids in liver PL from control mice (closed symbols) and mice intoxicated with bromobenzene (open symbols; same conditions as in panel A).

TABLE 2

MDA Release, Diene Conjugation Absorption, Amount of Carbonyl Functions and Decrease in Arachidonic and Docosahexaenoic Acids in Phospholipids of Liver Microsomes Incubated in an NADPH-Fe^{2+}-Dependent System

Time of incubation (min)	MDA (nmol/mg protein)	Diene conjugation absorption ($\Delta A_{233\ nm}$)[a]	Carbonyl functions (nmol/mg phospholipids)	Arachidonic acid (nmol/mg phospholipids)	Docosahexaenoic acid (nmol/mg phospholipids)
0	—	—	—	375 ± 49	189 ± 28
5	2.83 ± 0.97	0.158 ± 0.070	36.8 ± 1.8	323 ± 59	160 ± 31
15	7.48 ± 1.04	0.400 ± 0.035	43.1 ± 6.2	302 ± 32	143 ± 9
30	10.21 ± 0.50	0.817 ± 0.216	72.6 ± 14.2	237 ± 23	120 ± 14

Values reported are means ± SEM of 3 experiments. Control values (0 min of incubation) for MDA, diene conjugation absorption and carbonyl functions were subtracted from values for later times of incubation. Correlations among the parameters were as follows: MDA and diene conjugation absorption, $r = .967$, $P < 0.01$; MDA and carbonyl functions, $r = .940$, $P < 0.01$; carbonyl functions and diene conjugation absorption, $r = .941$, $P < 0.01$; MDA and loss of arachidonic acid, $r = -.959$, $P < 0.01$; MDA and loss of docosahexaenoic acid, $r = -.981$, $P < 0.001$.
[a]Phospholipid concentration: 1 mg/ml in cyclohexane.

measure lipid peroxidation in vivo with comparable reliability. However, the polyunsaturated fatty acid levels, due to their large dispersion, give somewhat less sensitive measures of lipid peroxidation in individual animals.

Table 2 shows the results of an in vitro study of lipid peroxidation of liver microsomes incubated in the NADPH-Fe dependent system. These experiments, essentially similar to those reported by several other investigators (45-48), were performed to examine, in an in vitro system, the relationships among the aforementioned methods to detect lipid peroxidation. As can be seen in Table 2, the correlations among the four methods examined showed high statistical significances, as in the case of in vivo lipid peroxidation.

The good agreement among the results obtained by the different methods to detect lipid peroxidation in vivo and in vitro not only assures good reproducibility, but also reaffirms confidence in each of the procedures. However, caution is needed in using the decrease of arachidonic acid as the sole index of lipid peroxidation, because conditions exist under which loss of polyunsaturated fatty acids is unrelated to lipid peroxidation, such as inhibition of the chain elongation-desaturation system, essential fatty acid deficiency and other dietary changes.

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Hepatic Lipid Abnormalities in a Chemical/Viral Mouse Model for Reye's Syndrome

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We have examined hepatic lipid profiles in a mouse model for Reye's Syndrome (RS) in which young animals are exposed to nontoxic doses of an industrial pesticide emulsifier and subsequently are infected with sublethal doses of mouse-adapted human Influenza B (Lee) virus (FluB). The purpose of this study was to determine whether liver lipid content was altered in the mice, the time course of any changes, and whether lipid changes were consistent with liver pathology. Neonatal mice exposed dermally to the emulsifier, Toximul MP8 (Tox), had significantly elevated levels of hepatic cholesterol, with otherwise normal lipid composition. Subsequent inoculation of the mice with FluB significantly increased mortality rate. The combined Tox + FluB treatment had several significant effects on liver lipids, including a transient increase in phospholipid (PL) content, a reduction in neutral glycerides and persistently high cholesterol levels. Abnormalities in fatty acid profiles included an apparent elevation in medium chain fatty acids and increased ratios of PL arachidonic to docosahexaenoic acids. Histologically, there was no evidence of fat accumulation in the liver; however, hepatic mitochondria had severe structural abnormalities characteristic of RS. These studies demonstrate that chemical-dependent enhancement of viral virulence is associated with significant alterations of hepatic lipids. We believe that these abnormalities are related to mitochondrial structural damage in RS despite the absence of hepatic steatosis.
Lipids 22, 217-223 (1987).

Reye's Syndrome (RS) is a potentially fatal disease of children that is characterized by abnormal serum lipid profiles (1-3), fatty infiltration of the liver (4-7), hepatic mitochondrial damage (8), hyperammonemia (9) and brain edema (9). Abnormalities in serum and hepatic lipids are believed to reflect the key metabolic defects in RS; however, the factors that lead to and perpetuate these abnormalities are not known. It has not been possible to study the time courses of development of lipid and other abnormalities in RS patients, since these changes occur either before and/or during an associated prodromal viral-like illness. Accordingly, it has become necessary to examine these changes using appropriate animal models.

We have demonstrated previously that suckling mice exposed dermally to nontoxic doses of an industrial pesticide emulsifier, Toximul MP8 (Tox), and subsequently infected with encephalomyocarditis virus (EMC) (10) or mouse-adapted human Influenza B (Lee) virus (FluB) (11) developed many of the features characteristic of RS, including hyperammonemia, abnormal urea-cycle enzyme activities and abnormal hepatic mitochondrial structure and function (Table 1). Recently we reported that combined Tox/FluB treatment also induced abnormal serum lipid profiles in the mice (12), which were manifested as hypopanlipidemia (including hypocholesterolemia), a feature of human RS. However, unlike in RS, the mice did not exhibit serum freefattyacidemia.

The purpose of this study was to examine hepatic lipid profiles in the mouse model to determine (i) whether there were quantitative and/or qualitative changes associated with the different treatment modalities (emulsifier alone, virus alone, emulsifier plus virus), and, if so, whether they were similar to those seen in patients with RS; (ii) the time course of evolution of these changes; and (iii) whether any abnormalities determined analytically were consistent with histological changes in the mouse livers.

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EXPERIMENTAL PROCEDURES

Chemical/viral mouse model. Details regarding the chemical/viral mouse model for RS have been described previously (11,12). Briefly, newborn Swiss white CFW mice (bred from animals obtained from Canadian Biobreeding Farms, St. Constant, Quebec, Canada) were pooled at 24 hr of age and randomly assigned to nursing females (7 pups/mother). Each mother and her pups were housed in a separate cage. Twenty-four hr later, half of the animals received abdominal applications of the industrial emulsifier Tox (Charles Tennant Co., Toronto, Canada; lot #9-30162) in corn oil (Tox/corn oil, 1:100, v/v); the control animals received corn oil alone. This application was continued daily for 10 days. For the first five days, 8.6 ± 2.6 mg of Tox (in solution) was painted on each mouse with a small brush; during the last five days the dose was increased to 25.8 ± 9.0 mg per animal. Forty-eight hr following discontinuation of dermal application (experimental day 13), half of each experimental group received intranasal inoculations of FluB under light ether anesthesia. The remaining animals received anesthetic alone. Deaths were recorded each morning throughout the experiment. On days 8, 12 (one day before viral infection), 15, 16 and 17, six live animals were removed from each of the four groups (control, Tox, virus, Tox + virus) for liver lipid analysis and morphological examination. Immediately after the animals were killed by decapitation, the livers were excised, wrapped in acetone-rinsed aluminum foil and flash-frozen for lipid studies. Samples for histological examination were prepared as described below. Each sample was coded, and samples were analyzed at random before being decoded.

Lipid extraction and analysis. Livers were homogenized and lipids extracted according to Folch et al. (13) with chloroform/methanol (C/M, 2:1, v/v) containing the antioxidant butylated hydroxytoluene (5 μg/ml). Lower phases were washed with 0.25 volumes of 0.88% KCl and then with methanol/H_2O (1:1, v/v). The lower organic phases were filtered, dried under a gentle stream of N_2 and

TABLE 1

Comparison of the Metabolic Abnormalities in Human Reye's Syndrome and in the Chemical/Viral Mouse Model

	Human Reye's Syndrome	Mouse model	
		Encephalomyocarditis virus	Influenza B (Lee) (mouse-adapted) virus
Liver fat	↑(−)	↑	−
necrosis	−	−	−
Mitochondrial enzyme activities	↓	↓	↓
Abnormal mitochondrial structure	+	+	+
Serum free fatty acids	↑	ND[a]	−
hypopanlipidemia	+	ND	+
Serum ammonia levels	↑	↑	↑
Intracranial pressure	↑	ND	↑
Noradrenaline	↓	ND	↓
Interferon	↓	↓	ND

[a]ND, not determined.

reconstituted in 1 ml C/M (2:1, v/v). Aliquots were taken for determination of phospholipid phosphorous (14) and cholesterol (Sigma Kit #351). The remains were dried under N_2, reconstituted in a small volume of solvent and spotted on a sheet of silicic acid-impregnated glass fiber paper (Gelman Scientific Co., Montreal, Ontario, Canada). Lipid classes were separated by instant thin layer chromatography (ITLC) using petroleum ether/diethyl ether/glacial acetic acid (90:10:1, v/v/v) as the solvent system. After drying, the paper was sprayed with 2′,7′-dichlorofluorescein (0.1%, by vol, in ethanol), and spots were identified under ultraviolet light by comparison with authentic standards. Areas corresponding to triglyceride (TG), free fatty acid (FFA) and phospholipid (PL) were extracted from the papers with chloroform, chloroform and C/M (2:1, v/v), respectively, with a recovery rate greater than 95%. A known amount of heptadecanoic acid (17:0) was added as the internal standard, and the samples were transmethylated with 10% BF_3 in methanol at 100 C for 45 min (15). Fatty acid methyl esters (FAME) were extracted with petroleum ether, extracts were dried under N_2 and the residues were reconstituted in small volumes of iso-octane (2,2,4-trimethylpentane). FAME were separated on a Sigma 3B gas liquid chromatograph (Perkin-Elmer, Ottawa, Ontario, Canada) equipped with a 30-m SP 2330 fused silica capillary column (Supelco Canada, Oakville, Ontario, Canada). The initial oven temperature (110 C for 3 min) was increased to 210 C at 3 C/min. Final temperature was held for 10 min. FAME were identified by comparison with authentic standards and quantitated by comparison with the internal standard, using an LCI 100 Laboratory Computing Integrator (Perkin-Elmer).

Fatty acid standards were purchased from Supelco Canada Ltd., Sigma Chemical Co. (St. Louis, Missouri) or Serdary Research Laboratories (London, Ontario, Canada). All solvents were high performance liquid chromatography grade.

Statistical analysis. Data represent the means of values obtained from a minimum of three animals. Data were analyzed using an analysis of variance (ANOVA) and differences were judged to be significant when $p < 0.05$.

Histological examination. Morphological examination was carried out on livers from control, Tox-, FluB- and Tox + FluB-treated animals to determine whether hepatic structure was consistent with the biochemical findings. We also examined the relationship between hepatic morphology and fat accumulation and the nature of the infecting virus, by comparing the morphological features seen with FluB with those determined in earlier studies with EMC (previously unpublished). The procedures used for oil-Red-O fat staining and electron microscopic analysis were described previously (11).

RESULTS

The animals were breastfed throughout the experiment, and milk was present in the stomachs of all at the time of death. There were no significant differences in body weights between the four experimental groups. As we have demonstrated previously (11), the group of neonatal mice given topical applications of Tox for 10 days and subsequently inoculated with FluB had a significantly higher mortality rate than did the other three experimental groups (Table 2). The reason for the deaths of four of the 33 control animals on day 16 is not known, as there are usually no deaths in either this group or the group that receives Tox alone. (Some deaths occur as a result of ether anesthesia; however, these are confined to the 24-hr period immediately following anesthetic administration.) Differences in mortality rates between the different experimental groups first became apparent on day 17 of the experiment, and by day 18, few animals had survived combined exposure to Tox + virus.

Total lipid content of the mouse livers (Fig. 1) was calculated as the sum of the PL and neutral lipid (TG, FFA, total cholesterol) fractions. Values obtained from day 12 of the experiment indicated that abdominal application of Tox for 10 days did not alter appreciably the total lipid content of the livers. On day 15, values for total lipid in the animals given Tox, FluB and Tox + FluB were significantly increased relative to control animals; however, the values obtained for the controls had fallen between days 12 and 15. This difference between control and treatment groups did not persist through day 17, the time when differences in mortality first became apparent (Table 2).

On day 15 of the experiment (48 hr after FluB and/or anesthetic), only those livers from mice treated with Tox alone exhibited increased levels of TG and FFA, and these values had returned to normal by day 17 (Fig. 2). Animals inoculated with virus alone did not have altered levels of these liver lipid components. However, the TG content of livers from mice that received combined Tox + FluB treatment was significantly lower than that of either control or Tox-treated animals at day 15. The reduction persisted to day 17, when values for TG in the combined treatment animals were significantly lower than all other treatment groups. Hepatic FFA content was not alterec.

TABLE 2

Cumulative Mortality Rates in Neonatal Mice Exposed to Tox and/or Infected with Mouse-adapted Influenza B (Lee) Virus and in Control Mice that Received Anesthetic Alone

	Experimental day (% survival)[a]			
Treatment group (N)	15	16	17	18
Control (33)	100	88	88	88
Tox (50)	100	100	100	100
Virus (47)	100	91	57	51
Tox + Virus (49)	100	96	37	12

[a]Data represent the percentage of animals in each group alive on the experimental day indicated, relative to the number (N) that survive the 24-hr period following virus inoculation and/or anesthesia.

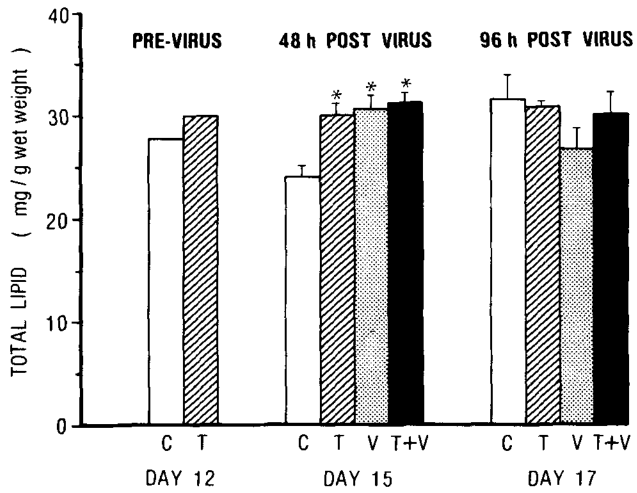


FIG. 1. Effects of Tox and/or FluB on liver total lipid content (mg/g wet weight) in the mouse model for Reye's Syndrome. Neonatal mice were given dermal applications of Tox (T) or corn oil (C) daily from experimental days 2 to 11. On day 13, half of the mice from each group received FluB under light ether anesthesia (V, T + V), and the other half received anesthetic alone. The animals were killed at the times indicated; livers were excised and lipids extracted and analyzed as described in the text. Data for total lipid are the sum of values obtained for the phospholipid, triglyceride, free fatty acid and cholesterol fractions. *, $p < 0.05$ relative to control animals.

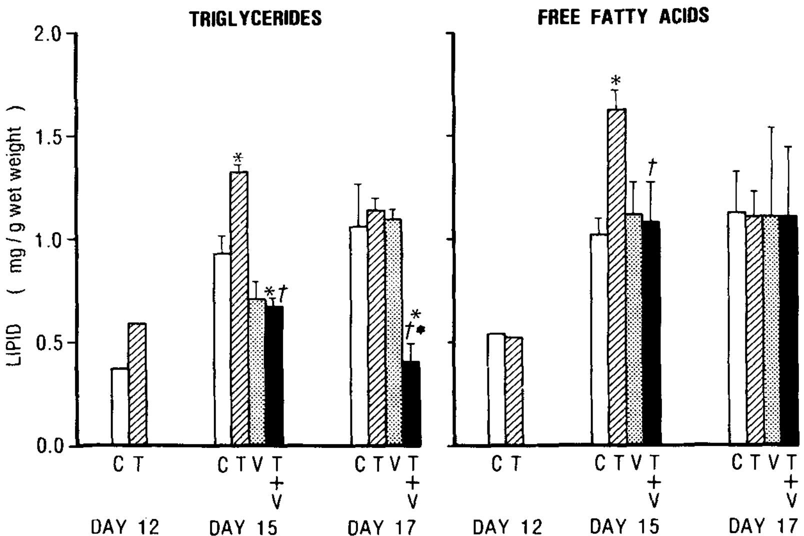


FIG. 2. Developmental changes in lipid class profiles in the mouse model for Reye's Syndrome. The experimental paradigm was as described in the legend to Fig. 1. Animals were killed on the days indicated, and livers from each of the four treatment groups were extracted. Triglyceride and free fatty acid fractions were separated and quantitated by gas liquid chromatography as described in the text. *, $p < 0.05$, relative to control animals; †, $p < 0.05$, relative to Tox-treated animals; ★, $p < 0.05$, relative to FluB-infected animals.

in FluB- or Tox + FluB-treated mice on any of the days examined.

PL constituted the major proportion (>85%) of the total lipid of livers from all four animal groups at each time point analyzed (Fig. 3). The only PL differences observed were increases at day 15 in livers from the three experimental groups; values had returned to normal by day 17.

Ten days' abdominal application of Tox resulted in a significant increase in the total cholesterol content of neonatal mouse livers (Fig. 4). Values for cholesterol gradually returned to normal in those animals that had received only Tox; however, levels were elevated on both days 15 and 17 in animals that received FluB, whether or not they had been preexposed to Tox.

We examined fatty acid composition of the PL, TG and FFA fractions of the mouse livers to determine whether shorter chain fatty acids (<C_{12}) accumulated under any of the experimental conditions and/or whether there was any evidence of abnormalities in the metabolism of hepatic polyunsaturated fatty acids. As shown in Table 3, there were apparent increases in the medium chain fatty acids in the TG and FFA fractions of livers from the Tox, virus and Tox + virus groups, with the greatest increase observed in the combined-treatment animals. They were not seen in all animals, which precluded statistical analysis. This probably reflects the fact that the analytical conditions were not optimized for quantitative recovery of these acyl constituents. Shorter chain fatty acids were not detected in the PL fraction of any animals.

Examination of the fatty acid composition of the liver PL revealed significant, time-dependent increases in the

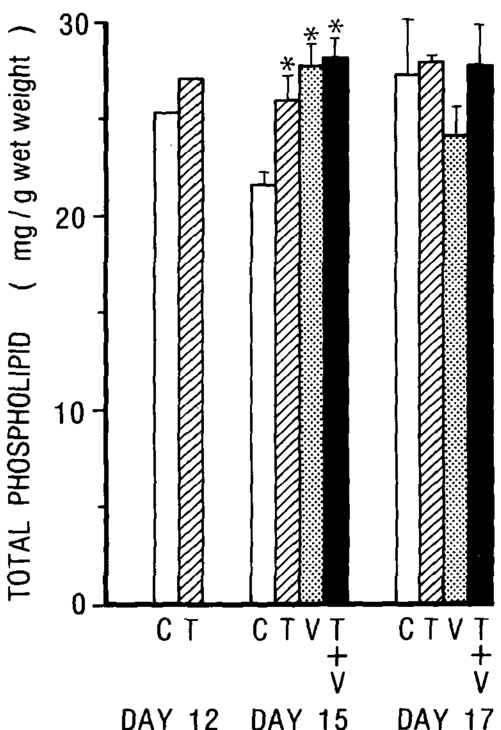


FIG. 3. The effect of chemical/viral treatment on liver phospholipid (PL) content in the mouse model. The experiment was carried out as described in the legend to Fig. 1. On the days indicated, livers were extracted and analyzed for PL phosphorus as described in Experimental Procedures. *, $p < 0.05$ relative to control animals.

TABLE 3

Fatty Acid Profiles of Hepatic Lipids in the Mouse Model

Fatty acids	Animal treatment group			
	C	Tox	V	Tox + V
Triacylglycerols				
8:0	—	6.1	6.8	6.4
10:0	—	1.5	4.2	7.4
12:0	1.7	1.9	2.1 ± 0.5	1.4
14:0	4.5 ± 0.7	5.4	4.8	3.8 ± 1.4
16:0	35.2 ± 1.1	30.9 ± 1.3	35.1 ± 2.1	31.8 ± 3.5
16:1n-7	2.8 ± 0.7	2.4 ± 1.0	3.8 ± 1.5	6.3
18:0	8.1 ± 3.2	5.5 ± 0.7	6.5 ± 0.7	8.0 ± 1.9
18:1n-9	22.2 ± 2.1	27.2 ± 3.9	23.4 ± 4.0	22.4 ± 1.0
18:2n-6	17.0 ± 2.7	18.3 ± 2.1	20.6 ± 3.9	14.5 ± 1.0
20:3n-6	0.9	—	—	—
20:4n-6	1.9 ± 0.2	1.35	1.4 ± 0.2	1.6 ± 0.4
20:5n-3	—	—	—	—
22:6n-3	3.2 ± 0.9	1.8 ± 0.2	3.1 ± 0.4	2.3 ± 0.5
Free fatty acids				
8:0	—	5.7	4.8	8.5
10:0	—	—	—	—
12:0	1.4 ± 0.6	1.1 ± 0.2	1.7 ± 0.3	2.0
14:0	2.8	3.4 ± 0.1	3.3	4.0
16:0	24.6 ± 2.2	24.4 ± 0.6	26.5 ± 3.0	27.9 ± 3.8
16:1n-7	4.0 ± 1.0	2.2 ± 0.1	1.7	2.6
18:0	6.1 ± 0.6	5.6 ± 0.8	6.5 ± 0.6	8.4 ± 2.3
18:1n-9	18.7 ± 2.9	24.8 ± 5.8	19.9 ± 2.6	22.9 ± 1.5
18:2n-6	18.3 ± 0.4	17.6 ± 2.3	19.2 ± 2.9	15.8 ± 1.3
20:3n-6	1.2	1.0	1.0	1.3
20:4n-6	5.8 ± 0.2	4.0 ± 0.7	6.3 ± 0.4	6.0 ± 0.6
20:5n-3	1.6 ± 0.4	—	1.5	1.9
22:6n-3	4.8 ± 0.7	3.7 ± 0.5	5.8 ± 0.3	5.6 ± 1.9

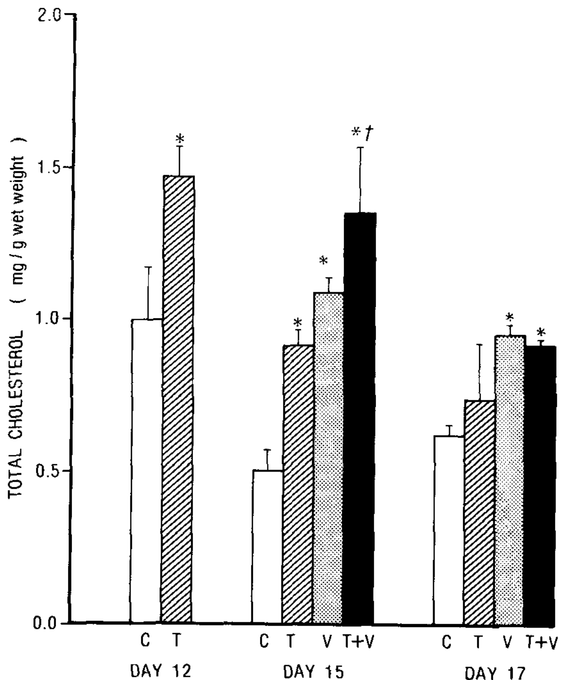


FIG. 4. Changes in liver cholesterol content in the mouse model. The experimental protocol is described in the legend to Fig. 1. Aliquots of the liver lipid extract were analyzed for cholesterol content as described in Experimental Procedures. *, $p < 0.05$, relative to control animals; †, $p < 0.05$, relative to Tox-treated animals.

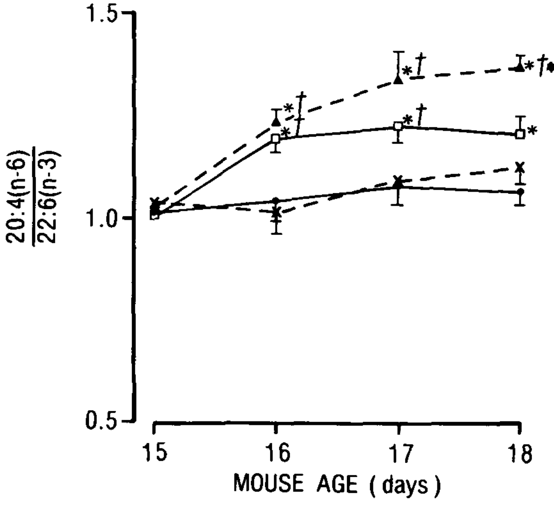


FIG. 5. Ratios of hepatic phospholipid (PL) arachidonic (20:4n-6) to docosahexaenoic (22:6n-3) acids in the mouse model for Reye's Syndrome. The experiment was carried out as described in the legend to Fig. 1. Lipid was extracted from livers from each of the four treatment groups (●, control; x, Tox; □, FluB; ▲, Tox + FluB) and the lipid classes were separated by instant thin layer chromatography. PL were transmethylated and fatty acid methyl esters were separated and identified by capillary gas liquid chromatography as described in the text. The data represent the means (± SEM) of the ratios of proportions of arachidonic (20:4n-6) to docosahexaenoic (22:6n-3) acids in the PL fraction. *, $p < 0.05$, relative to control animals, †, $p < 0.05$, relative to Tox-treated animals; ★, $p < 0.05$, relative to FluB-infected animals.

relative proportions of arachidonic (20:4n-6) to docosahexaenoic (22:6n-3) acids in the animals that received either FluB alone or Tox + FluB (Fig. 5). The altered ratios reflected increases in 20:4 and a decreased 22:6 content. By day 18 of the experiment, when few animals in the combined-treatment groups remained alive, the hepatic 20:4n-6/22:6n-3 ratio in these animals was significantly higher than that of any other group.

Light microscopic examinations were performed on 10 livers from each of the four treatment groups on day 17 of the experiment. Routine hematoxylin and eosin (H&E) stains revealed no evidence of necrosis, and oil-red-O staining showed no evidence of microvesicular fat accumulation (Table 4). This was in marked contrast to our earlier experiments in which treatment with the Tox and the natural mouse pathogen EMC resulted in panlobar hepatic microvesicular steatosis in 30–40% of the animals (Table 4).

Electron microscopic analysis of four livers from each group indicated that mice treated with Tox + FluB had severe hepatic mitochondrial structural abnormalities, including increases in the number and size of the mitochondria, with flocculation and focal rarefaction of their matrices (Fig. 6B). The mitochondrial cristae were detached and degenerated, with only a few recognizable abnormal cristae present. Similar mitochondrial abnormalities had been observed in our earlier studies with Tox + EMC exposure. The hepatocytic mitochondria of mice infected with FluB only or exposed to Tox only (Fig. 6A), showed mild pleomorphism, but there was no evidence of damage to their cristae or matrices. FluB was not detected in the livers of infected animals, and there was no evidence of cell inflammation or necrosis.

TABLE 4

Histologic Evaluation of Liver Tissue in the Mouse Model for Reye's Syndrome (Experimental Day 17)

Group	Fatty change[a]	Mitochondrial damage
FluB only	0[b]	0
Tox + FluB	0	3+
EMC virus only	0	0
Tox + EMC	0 to 3+ (40%)	3+

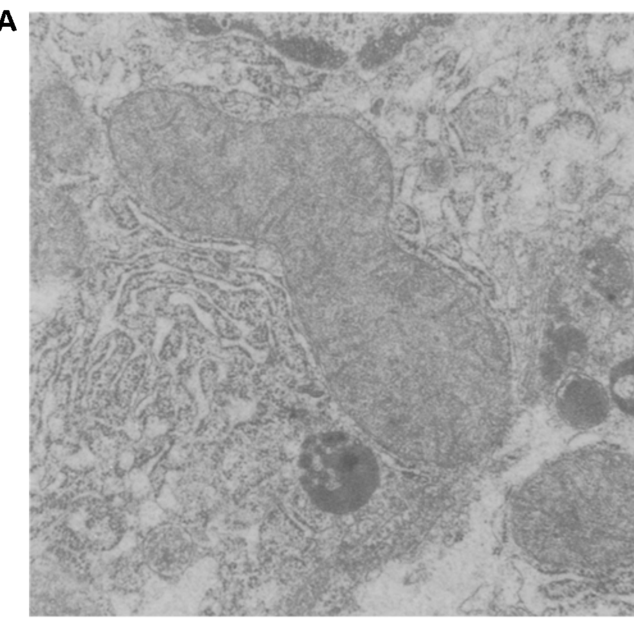
[a]Assessed with Oil-Red-O and electron microscopy.
[b]0, Absent; 3+, Severe.

DISCUSSION

Children who develop RS exhibit several prominent abnormalities in hepatic lipid metabolism, including increases in TG levels (4,5,17) generally accompanied by an apparent reduction in PL content (4,5,17). Reports of effects of the disease on hepatic FFA and cholesterol levels are inconsistent.

In our animal model for RS, suckling mice exposed to the industrial emulsifier Tox and subsequently infected with Influenza B virus showed no evidence of fatty infiltration of the liver by either chemical analysis (Fig. 1) or morphological examination (Fig. 6) at the time when the mortality rate in this group began to increase (Table 2). There are several possible explanations for the apparent lack of hepatic steatosis in the mouse. First, fat production may require specific virus-species interactions, and although FluB is one of the most common viruses

A



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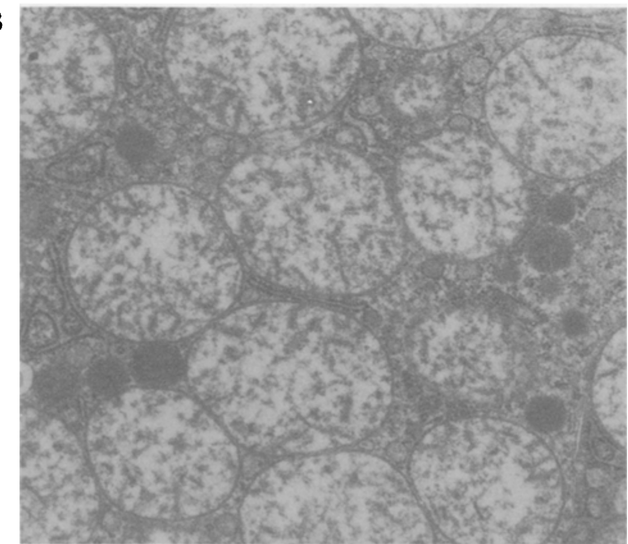

FIG. 6. The effects of chemical/viral treatment on the mitochondrial structure of neonatal mouse liver. On day 17 of the experiment (see legend to Fig. 1), livers were processed for electron microscopy as described in Experimental Procedures. The photomicrographs are of livers from animals that received (A) Tox or (B) Tox + FluB treatment. The liver from the combined treatment group shows marked swelling of mitochondria with extensive disintegration of the cristae. A few detached but still recognizable cristae are present. Focal rarefaction of the mitochondrial matrices and loss of dense bodies are evident. The micrographs show normal peroxisomes and no apparent lipid accumulation (30,000× magnification).

associated with development of RS in humans, it may not be appropriate for inducing fatty liver in animal models when the virus is not native to the species. This possibility is supported by the fact that hepatic steatosis did occur in our earlier studies with the mouse model, when the infecting pathogen was the native mouse virus EMC (10; Table 4). The constituents of the hepatic lipid obtained with Tox + EMC were not determined. A second possible explanation for lack of hepatic steatosis in our present model is that by taking samples for analysis 48 hr after viral infection, we may have missed a narrow time span during which levels were elevated. Our observation that hepatic lipid in the Tox, FluB and Tox + FluB groups were significantly higher (relative to control animals) in the samples analyzed at 48 hr (experimental day 15) suggest that levels may have been even higher in the first 24 hr following infection. However, TG levels were not elevated at any of the times tested, which is in contrast to the apparent increase in this fraction in patients with RS. Interestingly, several investigators have questioned the essentiality of a fatty liver in the pathogenesis of human RS (18,19); to our knowledge this question has not been fully resolved.

Perhaps one of the most important features of this study is the observation that, regardless of whether there was fat accumulation in animals given combined chemical/virus treatment, severe mitochondrial degeneration was seen consistently in this treatment group. Human RS is widely accepted to involve mitochondrial injury specifically, as reflected in morphological and biochemical anomalies of the liver and changes in blood chemistry (20). As is the case in humans, the mechanisms involved in mitochondrial injury in the Tox + FluB mice are unknown.

The mechanisms by which exposure to Tox and/or FluB altered PL, TG and cholesterol content in the neonatal mice are matters of conjecture at present. There have been few coordinated studies of the effects of viral infections on lipid metabolism in mammals, and the results of these have been complex and frequently contradictory in nature. Reports have documented inhibition (21-23) as well as stimulation (23,24) of lipid synthesis subsequent to viral infections. This probably reflects variations in the type of virus and the species infected; furthermore, changes can be transient in nature, with the result that data obtained may depend upon the duration of the infection (25). Clearly, liver lipid status at any given time reflects the net result of availability of precursors, relative synthetic and catabolic activities and intraorgan transport. For example, it has been suggested but not confirmed that decreased transport of TG from the liver due to impaired production of very low density lipoprotein is responsible for hepatic steatosis in RS patients (4). Our next goals are to determine whether enzymes involved in TG and cholesterol metabolism are altered as a result of the different treatment regimes and whether transport functions of the various lipid classes are abnormal in the model.

In addition to changes in lipid classes in the mouse livers, we observed distinct changes in fatty acid profiles (Table 3, Fig. 5). Other investigators have described virus-specific increases in the content of saturated (26,27) or unsaturated (28) fatty acids in host lipids. Changes in the proportions of these constituents may have deleterious effects on cell structure and dynamic properties, including the host's immune status (29). One consistent fatty acid modification that we observed was a significant time-dependent increase in the ratio of PL arachidonic (20:4n-6) to docosahexaenoic (22:6n-3) acids in the animals given combined Tox + FluB treatment (Fig. 5). Arachidonate is a precursor of the diene series of prostaglandins and leukotrienes, and increases in its proportions could alter

the synthesis and availability of these physiologically and pharmacologically active compounds, and as well, alter the concomitant production of superoxide anions. Abnormalities in arachidonic acid metabolism have been suggested to be involved in the pathogenesis of RS (3); however, whether these occur during the viral enhancement phase in the mouse model remains to be determined.

The apparent increase in the medium chain saturated fatty acids in the TG and FFA fractions of livers from the experimental animals is difficult to interpret, since they were not sufficiently consistent to allow statistical analysis. Nevertheless, their apparent increase could reflect impaired mitochondrial β-oxidation, which may be related closely to specific disorganization and dysfunction of this organelle (7).

One of the major questions raised by this study is whether there is a relationship between changes in cellular lipid content and viral enhancement. In the mice, the only significant effect of 10 days' exposure to Tox was an increase in hepatic cholesterol levels (Fig. 4), which was associated with a concomitant decrease in serum cholesterol (12). Reports of clinical studies have suggested generally that hepatic total cholesterol is not altered in patients with RS, although examination of the data in at least one report indicated that levels were elevated (17). Furthermore, serum cholesterol content is significantly reduced in RS (2), as it is in the mice. Interestingly, several groups of investigators have demonstrated that susceptibility to infection by and the virulence of several viruses, including Coxsackie-virus (30), Semliki Forest (31) and Sindbis (32) viruses increase with increasing membrane cholesterol content. Since we have not yet been able to isolate virus from the mouse livers, an association between Tox-dependent elevations in cholesterol and hepatic viral penetration appears unlikely. However, as suggested by Campbell et al. (30), it is possible that elevated cholesterol is related indirectly to defects in the nonspecific immune response, which are of primary importance in determining resistance to an invading pathogen. We have shown previously that interferon production is significantly reduced in mice exposed to the emulsifying agent (33).

In summary, we have found that neonatal mice given nontoxic topical applications of an industrial surfactant and subsequently infected with sublethal doses of virus develop a severe metabolic disease accompanied by an increased mortality rate and abnormal hepatic lipid metabolism. We have demonstrated a surfactant-dependent increase in hepatic cholesterol content, which may be related to increased susceptibility to viral infection (30–32). Once the animals are infected, there are distinct changes in liver lipid content, and depending upon the nature of the virus, there may be fat accumulation. However, even in the absence of hepatic steatosis, combined chemical/viral treatment of the young mice produces dramatic mitochondrial abnormalities that we believe to be closely related to the increased mortality associated with viral enhancement.

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Mass Spectra of the Picolinyl Esters of Isomeric Mono- and Dienoic Fatty Acids

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The picolinyl ester derivatives of the complete series of isomeric octadecenoates, methylene-interrupted octadecadienoates, and of octadec-9-ynoate have been subjected to gas chromatography-mass spectrometry. A fused-silica capillary column, coated with a cross-linked methyl silicone, was used for the separation. Electron impact spectra were determined at 70 eV. Earlier observations with a limited series of model compounds were confirmed, and it was shown that the picolinyl ester derivatives were of almost universal value in the location of double bonds in such isomers. Difficulties of interpretation arose mainly when the double bonds were close to the carboxyl group.
Lipids 22, 224–228 (1987).

Analysts have approached the problem of the location of double bonds in fatty acids by means of mass spectrometry (MS) in two ways (reviewed comprehensively elsewhere [1,2]): either the double bonds are fixed by adding some form of substituent group that gives a characteristic fragmentation pattern, or certain amide or related derivatives of the carboxyl group are prepared that appear to stabilize in the mass spectrometer the ions containing double bonds. The latter method is inherently more elegant and convenient, when it can be applied. Harvey, for example, showed that the picolinyl ester derivatives of certain model fatty acids gave characteristic spectra, which permitted unambiguous determination of the positions of double bonds (3–5). Derivatives of this kind, prepared from natural samples such as cod liver oil and pig testis lipids, were readily separated by gas chromatography (GC) on fused-silica capillary columns coated with nonpolar methyl silicone phases (6). GC-MS identification was accomplished for all components that were resolved and in sufficient abundance (>0.5% of the total fatty acids). However, the interpretations were based on a set of simple rules derived from spectra of a relatively limited number of model compounds in which the double bonds tended to be located in central positions in the fatty acyl chains. It is uncertain how applicable these rules are when double bonds are located at the proximal or terminal ends of the molecule. The electron impact mass spectra of the picolinyl ester derivatives of the complete series of isomeric *cis*-octadecenoic acids and of methylene-interruped *cis,cis*-octadecadienoic acids were therefore obtained, and they are described below.

MATERIALS AND METHODS

Most of the isomeric *cis*-octadecenoic acids, octadec-9-ynoic acid (7) and the isomeric methylene-interrupted *cis,cis*-octadecenoic acids (8) had been prepared earlier by total synthesis. 2-*Trans*-octadecenoic acid was a gift from F. D. Gunstone (Department of Chemistry, The University of St. Andrews, Scotland). The picolinyl ester derivatives were prepared as described previously (i.e., by reaction of the mixed anhydride of trifluoroacetic acid and the fatty acid with 3-(hydroxymethyl)-pyridine [6]). The GC-MS conditions were also as described previously (6). In brief, a fused-silica capillary column (25 m × 0.2 mm) coated with a cross-linked methyl silicone, with helium as carrier gas, was temperature-programmed from 60 C to 220 C at 50 C/min, then to 260 C at 1 C/min. The column outlet was connected directly to the ion source of a Hewlett-Packard 5970 mass selective detector, operated at an ionization energy of 70 eV.

RESULTS AND DISCUSSION

Picolinyl octadecenoates. Most of the fatty acids were more than 95% pure as judged by the total ion current trace from the mass spectrometer, and spectra were taken of the component eluting at the center of each peak, so the spectra should be representative. The spectra for each of the isomeric picolinyl octadecenoates are obviously distinctive; data for some of the ions of diagnostic value are listed in Table 1. The spectra for the 9- and 11-isomers resemble closely those published by Harvey, although his were obtained at an ionization potential of 25 eV, which was claimed to give better results (3). A definitive explanation for the discrepancy is not possible, but it may be related to differences in instrumentation. In each instance, a substantial molecular ion (M, m/z = 373) was observed, and the magnitude of the $(M-1)^+$ ion increased with the distance of the double bond from the carboxyl group. The base peak was generally at m/z = 92, but occasionally was at m/z = 93, representing an ion containing the pyridine ring and its methylene group. Other ions containing the pyridine ring at m/z = 108, 151 (cleavage between C2 and C3 of the aliphatic chain) and 164 (cleavage between C3 and C4 of the aliphatic chain) are almost always prominent. For example, the magnitude of the ion at m/z = 108 tends to increase with the distance of the double bond from the carboxyl group. The exception is the 2-isomer, where this ion is the base peak. Although this is the 2-*trans* isomer, none of the *cis* isomer being available, the configuration of the double bond is unlikely to affect the spectrum; certainly no difference was found between configurational isomers of the 9- and 11-octadecenoates (data not shown). The ion at m/z = 151 containing the picolinyl moiety is the base peak for the 4-isomer, and that at m/z = 164 is abundant for all but those isomers where the double bond is close to the carboxyl group.

In all the spectra, there are ions diagnostic for the positions of the double bonds. The points where characteristic fragmentations occur are shown for picolinyl octadec-9-enoate in Figure 1a. Doublets of prominent ions 14 atomic mass units (amu) apart representing cleavage on the distal side of the double bond were earlier found to be of special value in the identification of unknown fatty acids (6). In the spectrum of the 10-isomer illustrated in Figure 2 (as

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TABLE 1

Relative Abundances[a] of the Molecular Ion and of Ions Characteristic of the Picolinyl Moiety, and m/z Values[b] and Relative Abundances of Ions Characteristic of Double Bond Positions in Mass Spectra of Picolinyl Octadecenoates

Double bond position	m/z = 92	m/z = 108	m/z = 151	m/z = 164	M	M-1	Doublet		Gap of 26 amu		Gap of 40 amu
							A[c]	B	C	D	E (to C)
2	79	100	27	4	26	8	45(190)	40(177)			
3	100(93)	43	19	10	31	4	15(204)	8(190)			
4	84(93)	53	100	3	30	3	9(218)	2(204)			
5	100(93)	43	8	21	31	2	21(232)	4(218)	3(204)	1(178)	21(164)
6	100(93)	54	22	19	29	3	32(246)	5(232)	7(218)	1(192)	4(178)
7	100	70	16	52	52	8	43(260)	17(246)	4(232)	8(206)	5(192)
8	100	73	20	67	48	11	37(274)	28(260)	5(246)	10(220)	7(206)
9	100	75	22	61	37	7	35(288)	30(274)	10(260)	4(234)	10(220)
10	100	75	30	41	46	10	37(302)	39(288)	9(274)	2(248)	8(234)
11	100	72	36	46	43	9	32(316)	43(302)	10(288)	4(262)	7(248)
12	100	72	28	47	53	10	41(330)	49(316)	13(302)	4(276)	12(262)
13	100	67	28	50	50	10	33(344)	48(330)	13(316)	2(290)	11(276)
14	100	76	29	42	54	12	10(358)	52(344)	12(330)	3(304)	15(290)
15	100	88	38	59	54	12	12(372)	14(358)	18(344)	3(318)	17(304)
16	100	90	35	58	50	17		17(372)	5(358)	5(332)	28(318)
17	100	74	29	44	35	20			20(372)	1(346)	37(332)

[a]Expressed as a percentage of the base ion.

[b]In parentheses.

[c]Points of cleavage of ions A to E can be seen by reference to Figure 1a.

a

A B C D E

$CH_3(CH_2)_5CH_2CH_2CH{=}CHCH_2(CH_2)_6COOCH_2$–(pyridyl, N)

b

A B C D E

$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CHCH_2(CH_2)_6COOCH_2$–(pyridyl, N)

FIG. 1. The bonds where characteristic mass spectrometric fragmentation occurs for (a) picolinyl octadec-9-enoate and (b) picolinyl octadeca-9,12-dienoate.

noted above, spectra for the 9- and 11-isomers have already been published [3]), these ions are at m/z = 302 and 288. They were rationalized in terms of initial abstraction of allylic hydrogens on either side of the double bond with formation of a relatively stable conjugated diene system (3). These peaks are easily picked out in the mass spectra of most of the isomers. They are not, of course, seen in the spectra of those isomers where the double bond is close to the terminal methyl group, as shown in Figure 3, in which the spectrum of the 16-isomer is illustrated, because the proposed mechanism cannot operate here. The doublet is particularly pronounced with the 2-isomer, where a conjugated dienoic ion would also interact with the carbonyl function, but it is less prominent with the 3- and 4-isomers, as is evident from Figure 4, which illustrates the spectrum of picolinyl 3-octadecenoate. It is recognizable even with the latter isomers, however, as it is in regions of the spectrum where other ions are less abundant.

Distinctive fragmentation at the double bond is also apparent for each isomer. For example, to consider the 10-isomer (Fig. 2) once more, a regular series of ions 14 amu apart is seen in the high mass region, corresponding to fragmentations between each of the successive methylene groups, i.e., at m/z = 358 (M-CH_3), 344, 330, 316, 302, 288 and 274. There is then a gap of 26 amu to m/z = 248 (at points C and D on Fig. 1a), for the double bond carbons, followed by a further series of ions 14 amu apart at m/z = 234 (ion E in Fig. 1a), 220, 206, 192, 178 and 164. For some of the isomers, the gap of 26 amu is not always immediately obvious, but the gap of 40 amu between ions C and E is almost always readily identifiable. In addition to the other diagnostic ions, ions representing cleavage at C, D and E are evident in the spectra of the 5- to 17-isomers, as were the series of ions 14 amu apart that originate from either side of the double bond (other than from the 17-isomer, of course). The exceptions are the 2-, 3- and 4-isomers. With these, the fact that the double bond must be close to the carboxyl group can be recognized from the fact that only a single regular series of ions 14 amu apart is evident from the molecular ion downward, the doublet ions (A and B in Fig. 1a) being diagnostic and standing out from the surrounding ions. The 4-isomer can also be recognized because its base peak is at m/z = 151 rather than at m/z = 92, in addition to the doublet peaks (A and B).

Picolinyl octadec-9-ynoate. The spectrum of a single monoacetylenic ester was obtained, i.e., for picolinyl octadec-9-ynoate ("stearolate"). This resembled the spectrum of the corresponding monoene, in that there was a prominent doublet on the distal side of the triple bond (at m/z = 272 [45%] and 286 [46%]), and a gap of 24 amu from m/z = 258 (14%) to 234 (25%) for fragmentations on either side of the triple bond. The doublet ions may

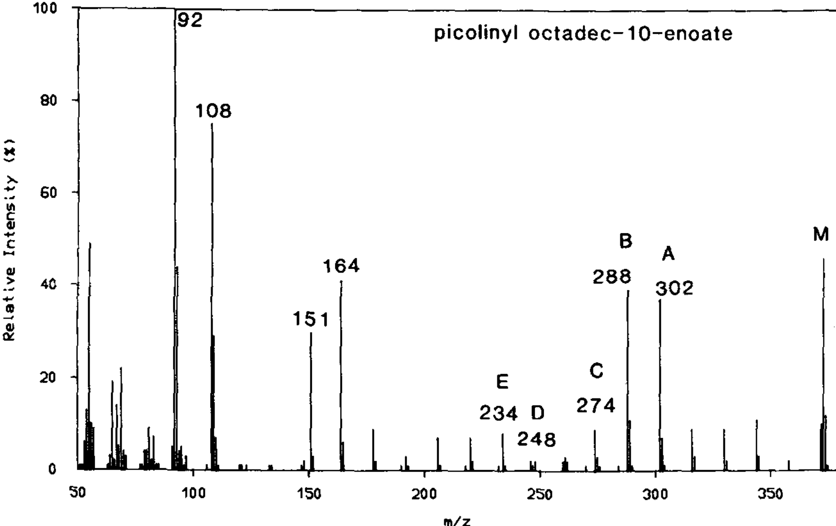

FIG. 2. The mass spectrum of picolinyl octadec-10-enoate. Ions A to E correspond to cleavage at the corresponding points relative to the double bond in Fig. 1a.

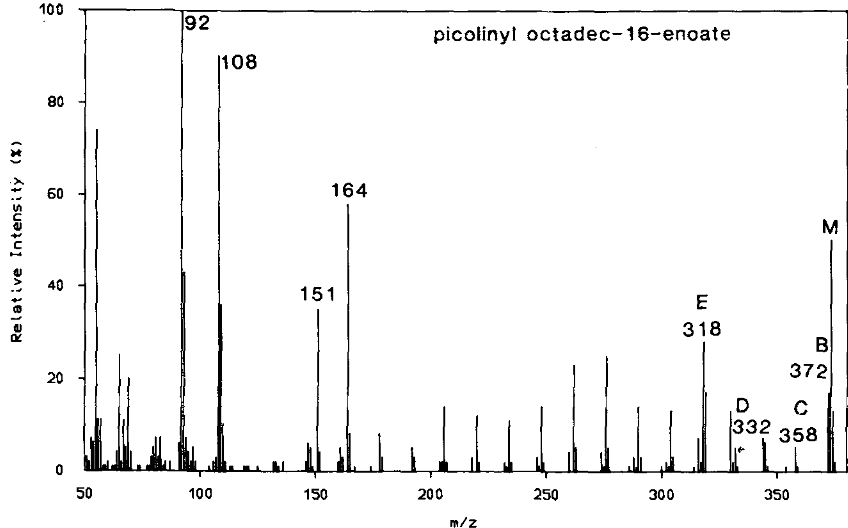

FIG. 3. The mass spectrum of picolinyl octadec-16-enoate. Ions B to E correspond to cleavage at the corresponding points relative to the double bond in Fig. 1a.

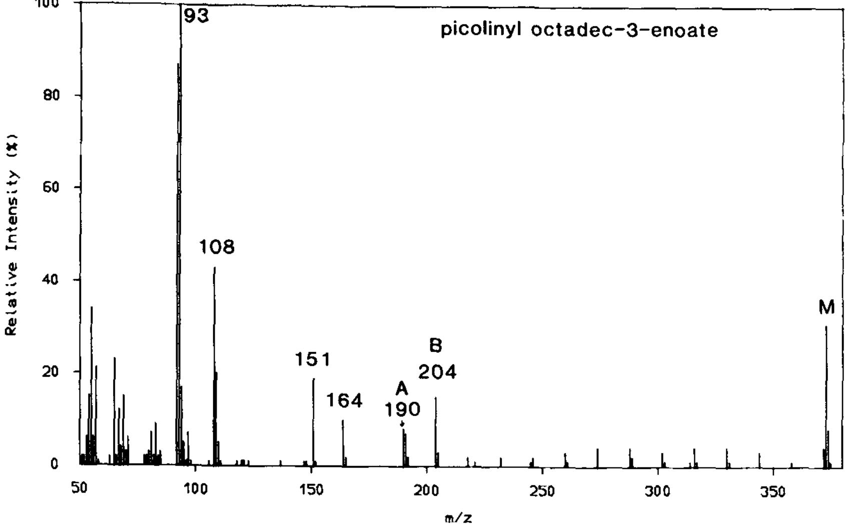

FIG. 4. The mass spectrum of picolinyl octadec-3-enoate. Ions A and B correspond to cleavage at the corresponding points relative to the double bond in Fig. 1a.

TABLE 2

Relative Abundances[a] of the Molecular Ion and of Ions Characteristic of the Picolinyl Moiety, and m/z Values[b] and Relative Abundances of Ions Characteristic of Double Bond Positions in Mass Spectra of Picolinyl Octadecadienoates

Double bond positions	m/z = 92	m/z = 108	m/z = 151	m/z = 164	M	M-1	Gap of 26 amu (terminal bond)		Gap of 26 amu (proximal bond)		Gap of 40 amu
							A[c]	B	C	D	E (to C)
2,5	100	75	34	3	72		1(202)				
3,6	100(93)	40	46	3	54	4	2(216)	48(190)	4(176)	46(151)	
4,7	100	74	53	12	59	5	2(230)	11(204)	1(190)	12(164)	53(151)
5,8	100(93)	49	13	17	58	6	2(244)	8(218)	2(204)	2(178)	17(164)
6,9	100	50	17	21	37	4	2(258)	8(232)	3(218)	3(192)	2(178)
7,10	100	55	15	43	66	8	8(272)	13(246)	9(232)	5(206)	4(192)
8,11	100	51	14	47	62	9	7(286)	12(260)	8(246)	5(220)	7(206)
9,12	100	58	15	39	47	7	5(300)	11(274)	11(260)	3(234)	6(220)
10,13	100	58	24	42	55	10	10(314)	14(288)	27(274)	4(248)	9(234)
11,14	100	59	20	41	57	13	9(328)	14(302)	32(288)	4(262)	10(248)
12,15	100	68	24	48	55	15	13(342)	13(316)	42(302)	5(276)	14(262)
13,16	100	67	22	48	31	10	2(356)	4(330)	32(316)	3(290)	13(276)
14,17	100	58	20	41	39	18	18(370)	1(344)	48(330)	2(304)	23(290)

[a]Expressed as a percentage of the base ion.
[b]In parentheses.
[c]Points of cleavage of ions A to E can be seen by reference to Figure 1b.

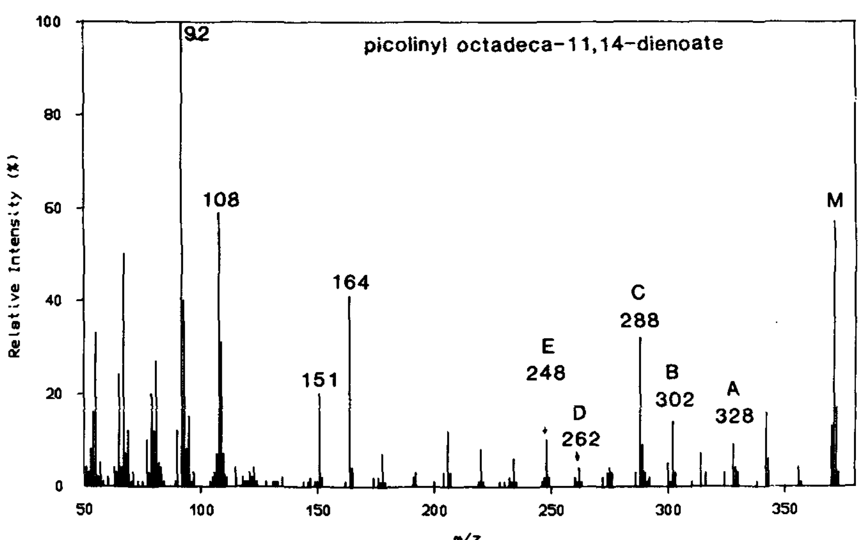

FIG. 5. The mass spectrum of picolinyl octadeca-11,14-dienoate. Ions A to E correspond to cleavage at the corresponding points relative to the double bond in Fig. 1b.

have arisen by abstraction of allylic hydrogens with formation of a conjugated double bond system, as with the monoenes (3). The ions characteristic of the picolinyl moiety are as expected. General rules on fragmentation cannot be derived from a single isomer, and the data are described simply as a guide for future work.

Picolinyl octadecadienoates. The ions in the mass spectra of the picolinyl 2,5- to 14,17-octadecadienoates of value for identification purposes are listed in Table 2. A substantial molecular ion (M) is always found (m/z = 371), and once more the abundance of the ion at $(M-1)^+$ tended to increase with the proximity of the double bonds to the terminal methyl group. As for most other picolinates, the base peak is at m/z = 92 (or 93), and the ions at m/z = 108, 151 and 164 are particularly prominent. Only for the 2,5- and 3,6-isomers is the ion at m/z = 164 less abundant.

There are also a number of ions that are diagnostic of the positions of the double bonds, and the positions of these are illustrated with reference to the naturally occurring 9,12-isomer in Figure 1b. As the spectrum of this compound has been published (5), that of the 11,14-isomer, which is also found in nature, is reproduced here in Figure 5 as an example of an isomer with centrally located double bonds. With the latter, a series of ions

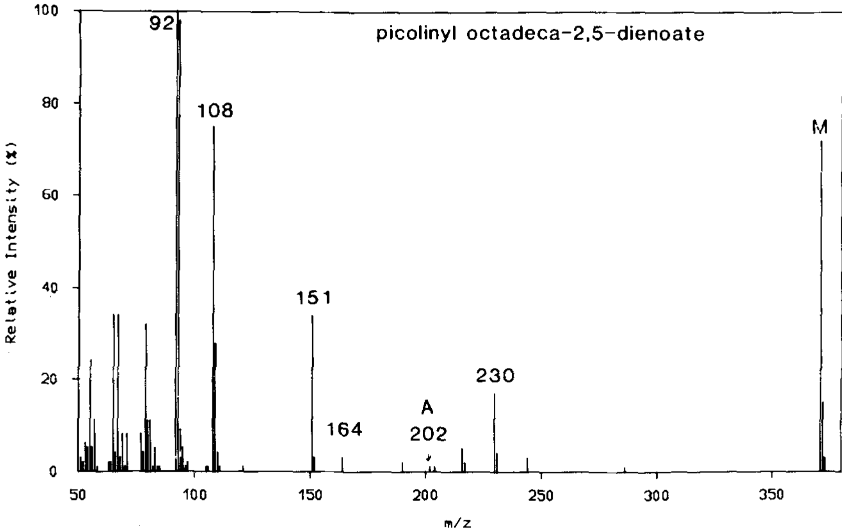

FIG. 6. The mass spectrum of picolinyl octadeca-2,5-dienoate. Ion A corresponds to cleavage at the corresponding point relative to the double bond in Fig. 1b.

14 amu apart is seen representing cleavage between successive methylene groups from the methyl end (at m/z = 356, 342 and 328 [ion A]). There is then a gap of 26 amu for the terminal double bond to m/z = 302 (ion B), followed by one of a further 14 amu to m/z = 288 (ion C) and a gap of 26 once more for the proximal double bond to m/z = 262 (ion D). As for the spectra of the monoenes, gaps of 40 amu between m/z 328 and 288 and between m/z = 288 and 248 (ion E) are usually easier to pick out than the gaps of 26 amu. A second series of relatively abundant ions 14 amu apart commences at m/z = 248 and continues until the carboxyl group is reached. Analogous ions to these are seen in the spectra of the picolinyl esters of the 3,6- to the 14,17-isomers, and ions A to E especially can be used to identify immediately the positions of the double bonds (as detailed in Table 2).

Only the spectrum of the 2,5-isomer defied immediate interpretation in these terms. It was remarkably devoid of ions in the high mass region, apart from the molecular ion itself, which is relatively more abundant than for any of the other isomers. There is an additional abundant ion at m/z = 230 (17%), which presumably represents cleavage between carbons 8 and 9 in the chain, although why such a fragmentation should be favored is not immediately obvious. The spectrum of this compound is illustrated in Figure 6 for reference purposes.

Conclusions. All the picolinyl esters were successfully chromatographed on a column of fused silica coated with a cross-linked methyl silicone phase. The retention times were determined over a period of several weeks, when small variations in the elution conditions could have occurred, so the data are not recorded here. On the other hand, it was apparent that for each series, the retention times follow a broadly sinusoidal relationship, very similar to that reported earlier for the methyl ester derivatives (9,10). Isomers with double bonds located approximately centrally are generally separable when these are 2 carbons apart (e.g., the 7-, 9- and 11-octadecenoates could be resolved). Such isomers are often found together in natural samples, as in those fractionated earlier (6). When the double bonds are near either end of the molecule, adjacent isomers are separable.

It is confirmed that the picolinyl esters of mono- and dienoic fatty acids are almost universally useful for locating double bonds. Problems arise in the interpretation of the mass spectra in terms of the positions of the double bonds only when they are close to the carboxyl group. Our experience has been that these derivatives are of equal value for polyunsaturated components, and fatty acids with up to six double bonds, methyl branches and as many as 24 carbon atoms have been separated and identified in this way.

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Characterization of Feline Omentum Lipids

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Feline omental lipid extracts, previously reported to be angiogenic in the cornea of rabbits, were fractionated and the major lipid components characterized. Approximately 97% of the chloroform/methanol extract consisted of triglycerides containing primarily 16:0, 18:0, 18:1 and 18:2 fatty acids. Trace quantities of free fatty acids, cholesterol, di- and monoglycerides were also detected. The phospholipid fraction, obtained by solvent partition and Unisil column chromatography and characterized by high performance liquid chromatography (HPLC)-mass spectrometry, was found to consist of phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine. The neutral glycolipids, isolated by solvent partition and Unisil column chromatography and identified by high performance thin layer chromatography and HPLC of their perbenzoylated derivatives, were found to consist of glucosyl- and galactosylceramides, galabiosylceramide, lactosylceramide, globotriaosylceramide and globotetraosylceramide. The complex glycolipid fraction, obtained from Folch upper phase solvent partition, was found to consist primarily of Forssman glycolipid and gangliosides GM_3 and GD_3. Smaller amounts of GM_1 and other unidentified gangliosides were also present.
Lipids 22, 229-235 (1987).

A lipid extract of feline omentum has been shown (1-6) to produce excellent angiogenic activity after a single injection into the central cornea of rabbits and to produce increased perfusion in a standardized wound as measured by in vivo nuclear imaging techniques with Technetium-labeled erythrocytes (7). Angiogenesis and chemical factors that mediate or inhibit the angiogenic processes have important implications in major diseases, and polypeptide and small molecular weight angiogenic factors have been purified from various pathological sources and from normal tissue in small quantities (9-18). Thus, tissues available in large quantities that contain angiogenic activity provide opportunities for isolation, structural analysis and extensive animal and clinical studies.

The lipid composition of omental tissue has received little attention. Rabinowitz et al. (19) reported on the lipid composition of human omental tissue, but only nonpolar lipids and phospholipids were studied. Neutral glycosphingolipids and particularly gangliosides have recently been shown to be associated with a variety of cellular responses and to modulate the activity of several growth factor receptors (20). The purpose of this study has been to determine the lipid composition of feline omentum and to assess the angiogenic activity of the lipid fractions with the chick embryo chorioallantoic membrane (CAM) assay. The CAM activity data will be presented elsewhere.

MATERIALS AND ANALYTICAL PROCEDURES

Materials. High performance liquid chromatography (HPLC) grade methanol and other reagent grade solvents and chemicals were obtained from Fisher Chemical Scientific (Fairlawn, New Jersey); Iatrobeads 6RS-8060 and 6RS-8010 were from Iatron Industries (Tokyo, Japan); DEAE-Sephadex (A-25) was from Pharmacia Fine Chemicals (Piscataway, New Jersey); Unisil was from Clarkson Chemical Co. (Williamsport, Pennsylvania); and high performance thin layer chromatography (HPTLC) plates were obtained from E. Merck (Darmstadt, Federal Republic of Germany). BondElut C18 reversed phase cartridges were obtained from Analytichem International (Harbor City, California). Ganglioside and glycolipid standards were prepared as previously described (21,22). Vibrio cholerae neuraminidase was obtained from Sigma Chemical Co. (St. Louis, Missouri).

Neuraminidase treatment of gangliosides. Purified gangliosides were dried under nitrogen, and 300 μl of 0.05 M sodium acetate buffer, pH 5.5, containing 0.025% $CaCl_2$ was added. Vibrio cholerae neuraminidase (100 μl, 0.1 units) was added and the sample was incubated for 3 hr at 37 C. The reaction was stopped by the addition of 2 ml of chloroform/methanol (C/M) (2:1, v/v); the mixture was placed over a C18 reversed phase cartridge (BondElut) and the nonlipid components were eluted with water. Any remaining gangliosides and lipid reaction products were eluted with methanol and C/M and examined by HPTLC.

Sugar and fatty acid analysis. For sugar and fatty acid analysis, the glycolipids were subjected to methanolysis in anhydrous 0.75 N HCl in methanol as described (23,24). The fatty acid methyl esters were analyzed by gas liquid chromatography (GLC) on an 8 ft × 2 mm ID coiled column packed with 3% OV-1 on 80-100 mesh Supelcoport with a Hewlett-Packard model chromatograph equipped with a flame ionization detector. The column oven was programmed from 160-250 C at 2 C/min. The methyl glycosides were analyzed as their trimethylsilyl derivatives on the same OV-1 column as described (25,26). For HPLC analysis of the lower phase neutral glycolipids, the fraction was perbenzoylated with benzoyl chloride in pyridine, and the benzoylated glycosphingolipids were separated and quantitated by HPLC on a column packed with Zipax with gradient elution and 230 nm detection as previously described (21). For direct probe mass spectrometry, glycolipid or ganglioside samples (5-50 μg) were trimethylsilylated by heating at 80 C for 60 min in 25 μl of pyridine/hexamethyldisilane/trimethylchlorosilane/N,O-bistrimethylsilyl/trifluoroacetamide (4:2:1:1:1, v/v/v/v/v). One to 5 μl of the sample was placed in a sample

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The abbreviations and nomenclature used for glycosphingolipids are those recommended by IUPAC (1976), *Lipids 12,* 455-468: GlcCer, glucosylceramide; GalCer, galactosylceramide; LacCer, lactosylceramide; $GaOse_2Cer$, galabiglycosylceramide, Gal(α1-4)Gal(β1-1)Cer; $GbOse_3Cer$, globotriglycosylceramide, Gal(α1-4)Gal(β1-4)Glc(β1-1)Cer; $GbOse_4Cer$, globotetraglycosylceramide, GalNAc(β1-3)Gal(α1-4)-Gal(β1-4)Glc(β1-1)Cer; $GalNAcGbOse_4Cer$, Forssman glycolipid. The ganglioside nomenclature is according to the system of Svennerholm (*J. Neurochem. 10,* 613-623, 1963).

cup, and the probe was heated from 100 to 440 C at a rate of 30 C/min. The mass spectra were obtained with a Finnigan model 4500 quadrupole mass spectrometer equipped with Teknivent, model 56K data system. It was operated with an ionizing current of 0.5 ma and an ionizing voltage of 70 eV. The ionizer temperature was 150 C. Repetitive scans of the mass range from 100 m/e to 950 m/e were acquired at 17-sec intervals.

Immunochromatography. Glycolipids were chromatographed on aluminum-backed HPTLC plates (Merck) with C/M/water (60:35:8, v/v/v), dried, then dipped in 0.05% polyisobutyl methacrylate in hexane as described by Brockhaus et al. (27). The plates were then soaked in phosphate-buffered saline (PBS) containing 1% bovine serum albumin for 2 hr before similar exposure to antibody for 2 hr at 4 C. The plate of upper phase neutral glycolipid was treated with Forssman monoclonal antibody IgM, purchased from American Tissue Culture Collection T1B 121. The TLC plates of the disialoganglioside fraction were treated with GD_3 monoclonal antibody IgM prepared in this laboratory. After washing in PBS, the plates were exposed to goat anti-mouse IgM conjugated to horseradish peroxidase for 2 hr at 4 C. After washing in PBS, the plates were developed with 33 mM 4-chloronaphthol in 0.02 M Tris-HCl buffer containing 20% methanol and 0.025% H_2O_2.

HPLC-mass spectrometry (LC-MS) of phospholipids. LC-MS of phospholipids was performed by methods developed in this laboratory (28). Briefly, HPLC was performed with a Spectra Physics (model 8700) gradient pump and a Brownlee cartridge, 6 cm × 2.0 mm ID, spherical silica (5 μm) column. The column was eluted with a linear gradient of solvent A, dichloromethane/methanol/water (93:6.5:0.5, v/v/v) and solvent B, dichloromethane/methanol/water/15 M NH_4OH (65:31:4:0.2, v/v/v/v), from 12% B to 45% B in 10 min and programming to 100% B in 2 min, at a flow rate of 0.5 ml/min. The sample to be injected was dissolved in dichloromethane/methanol/15M ammonia (1:1:0.01, v/v/v) at a concentration of about 1 μg phospholipid per 1 μl. The HPLC eluant was applied as a fine stream to the moving belt of a Finnigan LC-MS interface to achieve an even coating. The sample evaporator heater was at 330 C, and the cleanup heater was set at 210 C. A Finnigan model 4500 mass spectrometer was used with ammonia (0.75 torr) as the chemical ionization (CI) reagent gas. The ion source was at 150 C. Positive ion spectra were obtained by the system under the control of a Teknivent model 56K MS data system. MS data were collected from m/z 100–900 with 7-sec scans.

FRACTIONATION METHODS AND RESULTS

Fractionation of omentum extracts. Omentum from adult female cats were excised and minced as previously reported (6). The omentum preparations were homogenized in cold PBS and centrifuged at 4 C for 20 min in 2150-ml plastic bottles at 1,600 × g. After centrifugation, the lipid cake was removed and homogenized in 20 vol C/M (2:1, v/v). The extract was centrifuged at 200 × g for 10 min to remove particulate matter, and the clear supernatant was evaporated to dryness in vacuo to obtain the C/M fraction (CMFr), which was further fractionated as shown in Figure 1.

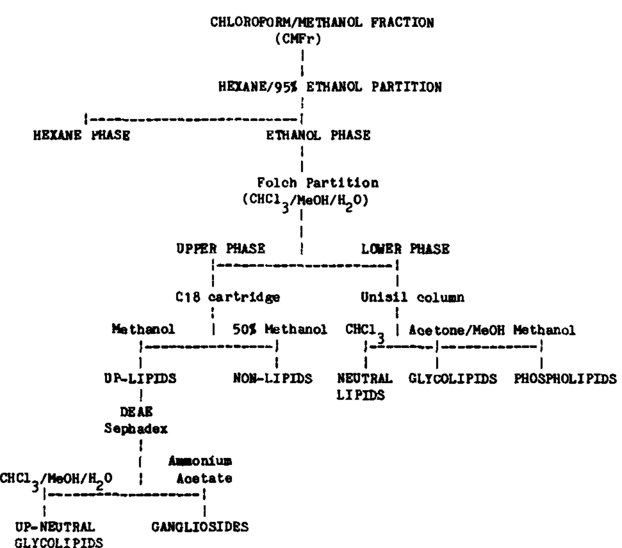

FIG. 1. Flow diagram for the fractionation of feline omentum chloroform/methanol extract.

TABLE 1

Weight Percent Distribution of Fractions from Feline Omentum Chloroform/Methanol Fraction (CMFr)

Fraction	Percent of CMFr	Percent of ethanol phase
CMFr	100	
Hexane	85	
Ethanol	15	100
Folch lower phase	13	86
Unisil column		
Neutral lipids	12	80
Glycolipids	0.03	0.17
Phospholipids	0.09	0.61
Folch upper phase	2	14
Nonlipid	0.27	1.9
Lipid		
Neutral glycolipids	0.18	1.25
Gangliosides		
Monosialo	0.007	0.05
Disialo	0.002	0.01
Polysialo	0.003	0.02

The CMFr was dissolved in hexane (ca. 60 ml/10 g of extract), and 0.66 vol of 95% ethanol was added. The phases were thoroughly mixed and allowed to separate. The lower phase was removed and the upper phase (hexane layer) reextracted with 95% ethanol. The lower phases were combined and reextracted with a fresh volume of hexane, and the hexane phases were combined. The phases were then taken to dryness in vacuo to obtain hexane upper phase material (hexane-UP) and ethanol lower phase material (ethanol-LP).

The hexane phase contained ca. 85% of the material in the CMFr and was shown to consist primarily of triglycerides as determined by HPTLC (weight distribution of the fractions from the omentum C/M extract is shown in Table 1). Alkaline methanolysis and gas chromatography

(GC)/MS analysis of the resulting fatty acid methyl esters revealed that 14:0 (1%), 16:0 (20%), 17:0 (1%), 18:0 (24%), 18:1 (44%) and 18:2 (10%) were the major triglyceride fatty acids. Small quantities of material migrating on HPTLC as free fatty acids, cholesterol, diglycerides and monoglycerides were also present in the hexane phase.

The ethanol-LP material was dissolved in C/M (2:1, v/v) (20 ml/g); 0.2 vol of water was added and phases were thoroughly mixed and allowed to separate as described by Folch et al. (29). The upper phase was removed, and the lower phase was washed with 0.4 vol of methanol/water (1:1, v/v). The upper phases were combined and both phases were taken to dryness in vacuo to obtain Folch-UP and Folch-LP material.

Folch-LP material was dissolved in chloroform and subjected to chromatography on a silicic acid (Unisil) column (30). The Unisil column was eluted successively with 20 column volumes of chloroform, acetone/methanol (9:1, v/v) and methanol to obtain the neutral lipid, glycolipid and phospholipid fractions, respectively. The neutral lipid fraction recovered from the Unisil column also consisted primarily of triglycerides, and small amounts of free fatty acids, cholesterol, di- and monoglyceride were detected by TLC analysis. The acetone/methanol glycolipid fraction was examined by TLC; components migrating as monohexosylceramides, lactosylceramide, globotriaosylceramide and globoside were present. Quantitative analysis of these glycolipids by HPLC is described below. The methanol phospholipid fraction was examined by TLC; components migrating as phosphatidylethanolamine, phosphatidylserine, phosphatidylcholine and sphingomyelin were present. Further characterization of phospholipids by LC-MS is described below.

HPLC analysis of the lower phase glycolipids. The lower phase glycolipids were examined by HPTLC with C/M/water (60:35:8, v/v/v) as the developing solvent and visualized with the orcinol spray reagent (31). They were also benzoylated with benzoyl chloride in pyridine, and the perbenzoylated derivatives were analyzed by HPLC with 230 nm detection as previously reported (32). The results are shown in Figure 2. These data show that the percent distribution of glycolipids in this fraction as GlcCer (Nfa), 26%; GalCer (Nfa), 9.6%; GlcCer (Hfa) + GalCer (Hfa) + $GaOse_2Cer$ (Nfa), 12%; LacCer, 11%; $GbOse_3Cer$, 10%; $GbOse_4Cer$, 26%.

LC-MS analysis of phospholipids. Aliquots of the phospholipid fraction were analyzed by LC-MS (28). Briefly, an aliquot containing at least 5 μg of each major phospholipid, was injected onto a column packed with spherical silica as described in Materials and Analytical Procedures. The column was eluted with a solvent gradient consisting of dichloromethane/methanol/ammonia with increasing concentrations of methanol and ammonia. The column effluent was applied to the Finnigan moving belt interface, solvent was removed under vacuum, and the chromatographic components were transported into the vaporization chamber and the lipids were volatilized into the ion source of the mass spectrometer. Positive ion mass spectra were continuously collected in the CI mode, with ammonia as the reagent gas. Figure 3 shows the total ion current chromatogram and selected ion plots specific for the phospholipid bases: serine (m/z 105), phosphoethanolamine (m/z 141) and phosphocholine (m/z 142). These data provide definitive identification and relative concentrations along with information on the fatty acid or ceramide composition of the different phospholipids. In order of decreasing concentrations, the following phospholipids were identified; phosphatidylcholine, sphingomyelin, phosphatidylethanolamine and phosphatidylserine. Trace quantities of phosphatidylinositol were also observed.

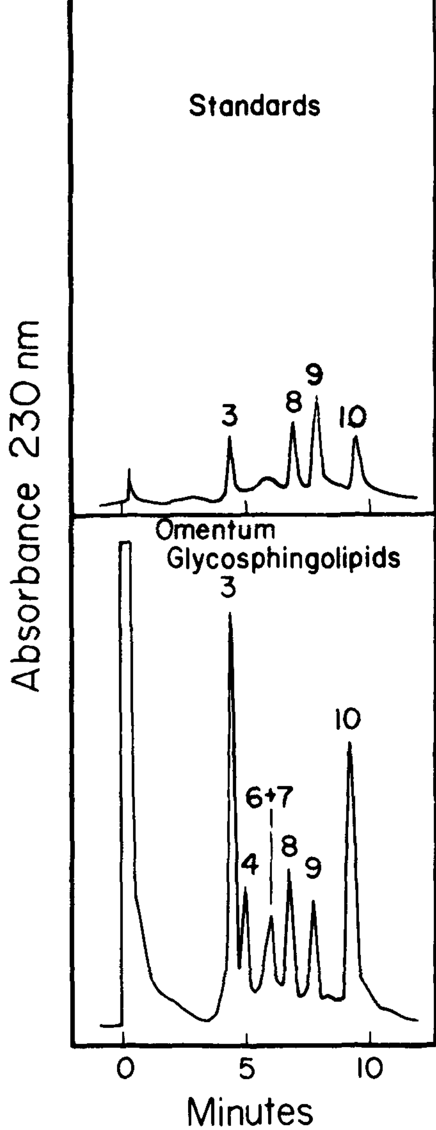

FIG. 2. High performance liquid chromatography of perbenzoylated neutral glycosphingolipids from feline omentum. A Zipax column with detection at 230 nm was used with a 13-min linear gradient of 1–20% dioxane in hexane at a flow rate of 2 ml/min. Peak numbers—3: GlcCer-NFA, glucosylceramide; 4: GalCer-NFA, galactosylceramide; 6: CMH-HFA, ceramide monohexoside with HFA (GlcCer and GalCer with hydroxyl fatty acids); 7: galabiosylceramide; 8: Lac-Cer, lactosylceramide; 9: CTH, ceramide trihexoside (globotriaosylceramide); 10: globotetraosylceramide (globoside).

Folch-UP material. Approximately 20% by weight of the ethanol-phase material was recovered in the Folch-UP. The Folch-UP was made 0.1 M with respect to KCl and applied to a C18 reversed phase cartridge (BondElut) (33), which was then washed with 4 vol of methanol/water (1:1, v/v) followed by elution with 4 vol of C/M (2:1, v/v).

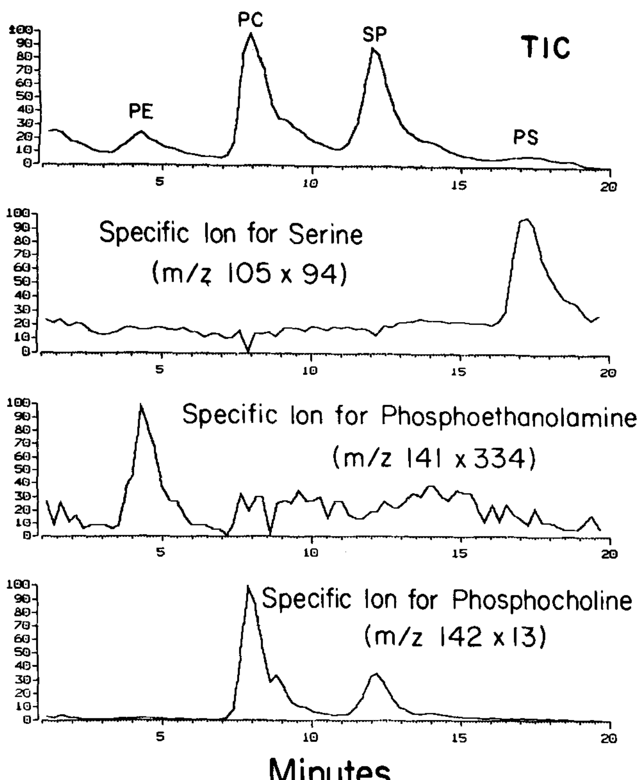

FIG. 3. Reconstructed plots of total ion current (a) and various specific ions monitored after high performance liquid chromatography (HPLC)-chemical ionization mass spectrometry (CIMS) of feline omentum phospholipids. An aliquot of the omentum phospholipid fraction equivalent to about 25 μg of total phospholipid was injected on a Brownlee silica gel (5 μm) cartridge HPLC column and eluted as described in the text. The eluate was applied to the moving belt of a Finnigan HPLC-MS interface. The solvent was removed under vacuum and the phospholipids were transported into the vaporization chamber and volatilized into the ion source (150 C) of the mass spectrometer. Positive ion mass spectra from m/z 100–900 were continuously collected every 7 seconds in the CI mode with ammonia as the reagent gas. The multiplication factor (*) for each plot is provided under each panel.

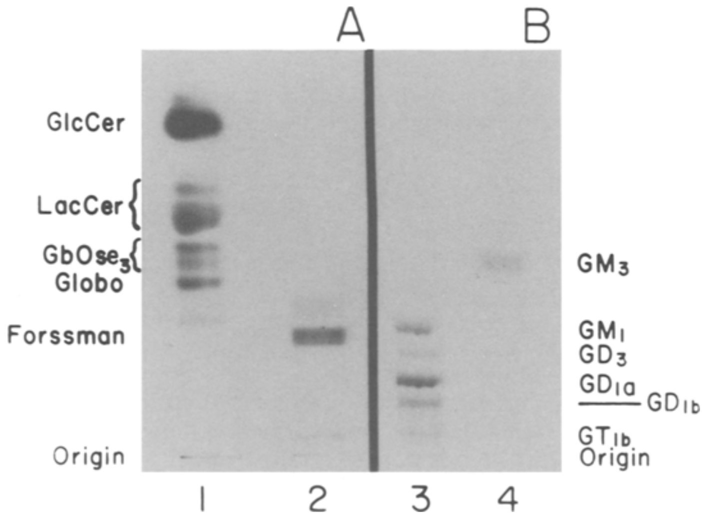

FIG. 4. High performance thin layer chromatography of feline omentum ganglioside and neutral lipid upper phase glycolipid fraction. Plate A was developed with chloroform/methanol/water (60:35:8, v/v/v), and the spots were visualized with orcinol spray reagent. The component migrating just above Forssman glycolipid is unidentified but migrates like paragloboside (nLcOse$_4$Cer). Plate B was developed with chloroform/methanol/0.25% $CaCl_2$ (55:45:10, v/v/v), and the spots were visualized with resorcinol spray reagents.

The two washes were separately collected and evaporated to dryness in vacuo to obtain nonlipid-UP material and lipid-UP material, respectively.

Nonlipid material. The nonlipid-UP fraction was taken to dryness and extracted with methanol. The majority of material was not methanol-soluble, and the suspension was centrifuged and the supernatant removed. The methanol-insoluble material was readily soluble in water. These fractions were examined by TLC, and the water-soluble fraction showed only one ninhydrin positive band. The bulk of this water-soluble material appeared to be salt. The methanol-soluble material contained at least six orcinol and ninhydrin positive components; GC/MS analysis, after trimethylsilylation, indicated this material was a complex mixture of sugars, amino acids, peptides and glycopeptides.

Lipid-UP. The lipid-UP material was dissolved in methanol and applied to a DEAE-Sephadex (acetate) column (31). The column was eluted with 10 vol of the same solvent to obtain the neutral lipid-UP fraction and then with 0.5 M ammonium acetate in methanol to obtain the ganglioside fraction. The fractions were evaporated to dryness in vacuo. The ganglioside fraction was desalted with a C18 reversed phase cartridge (BondElut) as described above.

Characterization of UP complex neutral glycolipids. The neutral lipid-UP fraction was found to constitute 40% of the lipid-UP material. Upon examination by HPTLC, this fraction was seen to consist primarily of an orcinol positive material migrating slightly slower than the globoside standard and small amounts of 3–4 more polar orcinol positive components (see Fig. 4). Immunoblotting with Forssman and SSEA-1 antibody indicated the major component was Forssman positive, and no SSEA-1 positive components were present. The major component was further purified by chromatography on an Iatrobead column (1 × 50 cm, 60 μ) eluted with hexane/isopropanol/water mixtures (23,26) and subjected to methanolysis and component analysis by GC/MS. Hexose ratios were found to be Glc/Gal/GalNAc (1.0:1.9:1.9). The fatty acids present were primarily 16:0, 18:0, 20:0, 22:0 and 24:0 with small amounts of the monounsaturated analogs, as shown in Table 2. The purified Forssman glycolipid appeared as four distinguishable bands when examined by HPTLC; these bands were isolated by preparative HPTLC and shown to contain different fatty acids, with the lowest band containing almost exclusively 16:0 and 18:0 fatty acids. The intact glycolipid was also silylated and examined by direct probe mass spectrometry. The spectra, shown in Figure 5, indicated the presence of terminal hexosamine, internal hexose residues, the presence of C18 sphingosine and fatty acids consistent with the data shown in Table 2. Taken together, these data indicate that the glycolipid is the Forssman pentaglycosylceramide (GalNAcGbOse$_4$Cer). Although position and configuration of linkages have not been directly determined, the antibody reactivity and glycolipid analytical data strongly support this structure.

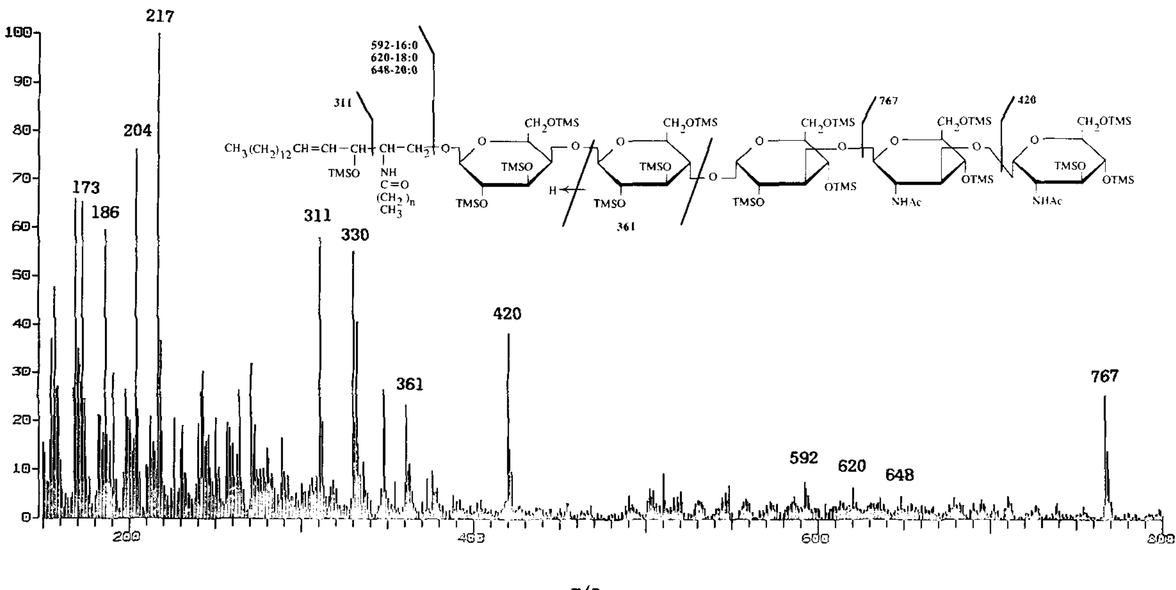

FIG. 5. Direct probe mass spectra of silylated pentaglycosylceramide from cat omentum extract. The signal at m/z 420 indicates the presence of terminal hexosamine while m/z 361 indicates internal hexose residues. The absence of an ion at m/z 451 indicates that no terminal neutral hexose is present, and the ion at m/z 767 is indicative of a terminal disaccharide consisting of two N-acetylhexosamine residues. These data are consistent with the presence of three neutral hexose and two hexosamine residues as in Forssman glycolipid (GalNAcGbOse$_4$Cer). The presence of C-18 sphingosine is indicated by the m/z 311 ion. Ions at m/z 592, 620 and 648 are indicative of 16:0, 18:0 and 20:0 fatty acid containing ceramides, respectively. These data are consistent with the fatty acid composition obtained by methanolysis and gas chromatography/mass spectrometry analysis of the methyl esters, as shown in Table 2.

TABLE 2

Percent Distribution of Fatty Acids in the Major Glycosphingolipids from Feline Omentum

Fatty acids	Forssman glycolipid	GM_3	GD_3
16:0	25.5	14.0	12.9
18:1	11.8	6.3	8.5
18:0	11.2	11.8	5.9
20:0	9.4	17.1	3.4
22:0	8.0	13.9	6.9
23:0	5.0	9.5	—
24:1	9.6	14.0	46.0
24:0	10.2	12.6	16.4

Only fatty acids present at concentrations greater than 2% are reported.

Characterization of omentum gangliosides. Examination of the ganglioside fraction by HPTLC revealed the presence of resorcinol positive components migrating as GM_3, GD_3 and minor amounts of GM_1, GD_{1a} and other polysialoganglioside components (see Fig. 4). The ganglioside fraction was treated with 0.25 N sodium hydroxide in methanol for 2 hrs at 37 C, neutralized with glacial acetic acid and desalted with a C18 reversed phase cartridge (Bond Elut). The alkali-treated ganglioside fraction was then subjected to chromatography on a DEAE-Sephadex column and eluted with 0.02 M, 0.08 M and 0.5 M ammonium acetate in methanol to obtain mono-, di- and polysialoganglioside fractions, respectively (24). The ganglioside fractions were separated into individual components by chromatography on a 0.4 × 50 cm, 10 μM particle, Iatrobead column eluted with C/M/water (65:35:8, v/v/v). Fractions of 1.2 ml were collected, aliquots were examined by HPTLC and fractions containing single components were appropriately pooled.

The monosialoganglioside fraction was shown by HPTLC to consist primarily of components migrating as a triplet of bands corresponding to the mobility of the GM_3 standard and a small amount of material migrating as GM_1. The monosialoganglioside fraction was further purified by chromatography on an Iatrobead column, and the fractions containing only components migrating as GM_3 were pooled. This material was treated with neuraminidase, and the lipid product was characterized as lactosylceramide by HPTLC and direct probe MS. The liberated sialic acid was shown by GC to consist only of N-acetylneuraminic acid. The intact ganglioside was subjected to methanolysis, and the sugars and fatty acids were examined by GC. The ratio of Glc/Gal was found to be 1.0:0.96; the fatty acids consisted primarily of 16:0, 18:0, 18:1, 20:0, 22:0, 23:0, 24:0 and 24:1, as shown in Table 2. The preparation was also examined by direct probe MS as the trimethylsilyl ether derivative. A mass spectra similar to that given by ganglioside GM_3 standard, sialyl(α2-3)galactosyl(β1-4)glucosyl(β1-1)ceramide, was obtained.

The disialoganglioside fraction was shown by HPTLC to consist primarily of a component migrating as GD_3. This material was further purified by chromatography on an Iatrobead column, and the fractions containing only a single component migrating as GD_3 were pooled. The preparation was subjected to methanolysis, and the methyl glycosides and fatty acid methyl esters were examined by GC/MS. The ratio of Glc/Gal was found to be 1.0:1.0, and the major fatty acid components were 16:0, 18:0, 18:1, 24:0 and 24:1, as shown in Table 2. The

material was treated with neuraminidase, and the lipid product was identified as lactosylceramide by HPTLC and direct probe MS. The liberated sialic acid was shown to consist only of N-acetylneuraminic by GC. Direct probe MS of the TMS derivative gave spectra consistent with GD_3. The material was also shown by immunoblotting to react with a monoclonal antibody prepared in this laboratory with demonstrated reactivity with GD_3.

The polysialoganglioside fraction was shown to contain components migrating on HPTLC as ganglioside GD_{1a}, GT_{1b}, but insufficient quantities were obtained for further analysis.

DISCUSSION

Omental tissue consists to a large extent of white adipose fat cells embedded in an extracellular matrix of collagen and elastin. According to Hodel (34), the fat droplets of the omental fat cells can reach diameters of 100 μm in well-fed animals. Quantities of macrophages are also usually present, sometimes visible grossly as "milky spots" (34-36). The cell types found in the omentum and especially in milky spots include fibroblasts, reticular cells, mesenchymal cells, histiocytes, macrophages, lymphocytes, plasma cells, mast cells, eosinophilic leukocytes and polymorphonuclear leukocytes (37). The tissue has long been known to be highly responsive to injury and infection (38,39). The studies by Rabinowitz et al. (19) on the lipid composition of human omentum showed that patients under caloric restriction showed a reduction in their total triglyceride content, a reduction in the content of unsaturated fatty acyl groups and a relative increase in phospholipid contents. Other factors such as injury and infection must also affect the lipid composition of this tissue. Species differences also exist, as we have shown in studies to be reported elsewhere, that the complex neutral glycolipids and gangliosides of porcine and bovine omentum are distinctly different from those found in this study of feline omentum.

The data reported here are the first on feline omental lipids and omental glycolipids. The triglyceride fatty acid composition of feline omentum resembled that reported by Rabinowitz et al. (19) for their control human omental tissue, except for the higher content of stearic acid. The phospholipid species found and their relative concentrations in the feline tissue also closely paralleled those reported for human tissue. The significant amounts of glycolipids found in the feline omental tissue are of interest. The presence of mono- and diglycosylceramides and globoseries glycolipids as well as gangliosides could possibly relate to the biological activity observed for the lipid extracts. Although some glycolipids may be characteristic of omentum, it is clear from ongoing studies of porcine and bovine that the glycolipid patterns are species-specific. It is anticipated that the identification of the lipid species reported here will be useful in characterizing the biologically active components. Recently Niinikoski et al. (40) reported that a hexosylceramide fraction from calf blood exerted an accelerating effect on wound-healing angiogenesis, and Gullino (41) reviewed reports on factors that can trigger angiogenesis and provided evidence that heparin, fibronectin and gangliosides are involved in the mobilization of capillary endothelium.

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Phospholipid Studies of Marine Organisms: 14. Ether Lipids of the Sponge *Tethya aurantia*[1]

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The novel unesterified alkyl glycerol monoethers, (2S)-1-(hexadecyloxy)-2,3-propanediol (*1*), (2S)-1-(16-methylheptadecyloxy)-2,3-propanediol (*2*) and (2S)-1-(15-methylheptadecyloxy)-2,3-propanediol (*3*) were isolated from the marine sponge *Tethya aurantia* and were characterized by spectroscopic methods. These three saturated ethers as well as a series of alk-1′-enyl glycerol monoethers were also encountered in the phospholipids of the same sponge after reduction with $LiAlH_4$. Incorporation experiments with dissociated cells of *T. aurantia* indicated that [1-^{14}C]-hexadecanol was incorporated into the unesterified alkyl glycerol monoethers.
Lipids 22, 236–240 (1987).

Sponges are the most primitive of the multicellular animals and have been found to be rich sources of many unusual compounds (1,2), including lipids (3). Recently, a number of reports have appeared on the direct isolation of alkyl and alk-1′-enyl glycerol monoethers from marine sponges (4–7). To date, such compounds have only been isolated in the unesterified form from marine sponges, although glycerol ethers in the esterified form are commonly encountered in a wide variety of organisms (8).

Our interest in *Tethya aurantia* stems from studies currently underway in this laboratory concerning the biosynthesis of unusual fatty acids found in this sponge (9). Here we report the direct isolation of novel alkyl glycerol monoethers from *T. aurantia* without any prior hydrolysis. Alkyl and alk-1′-enyl glycerol monoethers were also found in the phospholipids of *T. aurantia* after reduction with $LiAlH_4$. Incorporation experiments utilizing [1-^{14}C]-hexadecanol were carried out to establish whether the glycerol monoethers were of dietary origin or were biosynthesized in the sponge. The possible role of the unesterified alkyl glycerol monoethers is also discussed.

MATERIALS AND METHODS

Materials. [1-^{14}C]Hexadecanoic acid was obtained from Amersham Corp. (Arlington Heights, Illinois) and 1-O-hexadecyl-glycerol from Sigma Chemical Co. (St. Louis, Missouri).

General methods. T. aurantia was collected from Carmel River Beach, Carmel, California, in February and April, 1986, at depths of 5 to 10 m. After freeze-drying, the sponges were extracted by the method of Folch et al. (10), and the resulting lipid extract was fractionated by flash chromatography (11) by elution with hexane/ether mixtures, acetone and methanol. The unesterified alkyl glycerol monoethers, which were eluted in the ether fraction by flash chromatography, were purified further by normal phase high performance liquid chromatography (HPLC), which was performed on a Waters Associates HPLC system (M-45 pump, R-401 differential refractometer) using an Altex Ultrasil-Si column (25 cm × 10 mm ID) with hexane/ether (3:7, v/v) as the mobile phase at 2 ml/min. The methanol-eluting fraction from flash chromatography, containing the phospholipids, was reduced with $LiAlH_4$ as described previously (12). The resulting mixture, which consisted predominantly of fatty alcohols and glycerol ethers, was separated by normal phase HPLC as described above.

^{1}H nuclear magnetic resonance (NMR) spectra were recorded on a Nicolet NT 300 WB (300 MHz) spectrometer using 5 mm ID spinning sample tubes. All samples were recorded in $CDCl_3$ and are expressed as ppm downfield from tetramethylsilane (TMS), the primary reference being chloroform, which resonates at 7.26 ppm relative to TMS.

Gas chromatography/mass spectrometry (GC/MS) analyses were performed with either a Ribermag R10-10 quadrupole mass spectrometer connected to a Carlo Erba series 4160 Fractovap chromatograph or a Hewlett Packard 5970 quadrupole mass spectrometer coupled to a Hewlett Packard 5890 gas chromatograph.

The identity of the alkyl glycerol ether hydrocarbon side chains (Fig. 1) was determined by GC/MS of both the nitrile and the pyrrolidide derivatives generated from the corresponding alkyl iodides after cleavage of the ether linkage. The cleavage was accomplished by refluxing the alkyl glycerol ethers with concentrated hydriodic acid for 3 hr (13) and by converting the resulting iodides to the nitriles by heating for 30 min at 90 C with NaCN in dimethylsulfoxide (14). The nitriles were hydrolyzed by refluxing in 6% KOH/ethanol for 16 hr, and the resulting acids were transformed into the methyl esters by refluxing with HCl/methanol for 30 min. The pyrrolidides were then synthesized by heating the methyl esters with pyrrolidine/acetic acid (10:1, v/v) at 100 C for 30 min (15).

The identity of the hydrocarbon side chains of the alk-1′-enyl glycerol monoethers (Fig. 2) was established by GC/MS of the resulting pyrrolidide derivatives after hydrolysis of the alk-1′-enyl ether linkage and derivatization. The vinyl ether linkage was hydrolyzed with

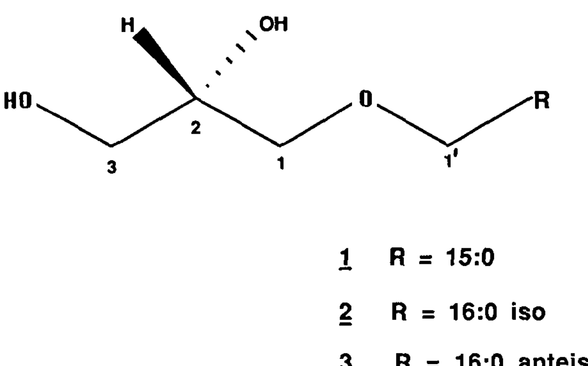

FIG. 1. Structures of the alkyl glycerol monoethers isolated from *T. aurantia*.

[1]For preceding paper, see Ayanoglu, E., Düzgünes, N., Wijekoon, W.M.D., and Djerassi, C. (1986) *Biochim. Biophys. Acta 863*, 110–114.

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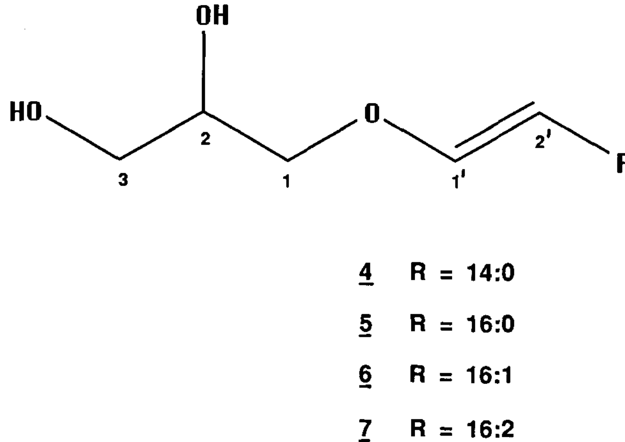

FIG. 2. Structures of the alk-1′-enyl glycerol monoethers isolated after reduction of *T. aurantia* phospholipids.

concentrated HCl for 2 min (16), and the resulting aldehydes were oxidized to the corresponding acids with Jones reagent (17). The methyl esters and pyrrolidides were synthesized from the resulting acids as described above.

Gas liquid chromatography (GLC) was carried out using a Hewlett Packard (HP) 5790 Series gas chromatograph equipped with an HP Ultra 2 (5% Ph Me Silicone) capillary column (25 m × 0.2 mm), temperature-programmed from 170 to 300 C at 5 C/min. Peak areas were calculated using an HP 3392A integrator.

Infrared spectra were recorded on a Perkin-Elmer 1310 infrared spectrophotometer.

Incorporation experiments. [1-^{14}C]Hexadecanol was synthesized byLiAlH$_4$ reduction of [1-^{14}C]hexadecanoic acid. Biosynthetic incorporation experiments were carried out using both intact sponges and dissociated sponge cells. [1-^{14}C]Hexadecanol (10 μCi in 0.25 ml 95% ethanol) was incorporated into intact sponges (ca. 6 cm in diameter) by injection and aquarium incubation in a minimum volume (ca. 200 ml) of seawater with aeration for 24 hr. The aquarium was then flushed continually with filtered seawater, with aeration, for a further 6 or 16 days, after which time the sponge was processed.

Dissociated sponge cells were produced by initially cutting the sponge into small pieces (ca. 1 cm × 1 cm), which were then pressed through a fine nylon cloth. To the filtrate (ca. 75 ml) was added [1-^{14}C]hexadecanol (10 μCi in 0.25 ml 95% ethanol), and the cell suspension was incubated in a 250-ml flask closed with a cotton plug, with shaking at 15 C for 16 hr. Isolation and analysis of the glycerol ethers were carried out as described above. Small aliquots (usually 1/100 or 1/50) of the ^{14}C samples were dissolved in 10 ml of organic counting scintillant, and the radioactivity was measured with a Beckman LS 7500 liquid scintillation counter. All results were corrected for background radiation and calculated as dpm by using a ^{14}C standard solution.

Identification of compounds: unesterified alkyl glycerol monoethers (Fig. 1). ^{1}H NMR (300 MHz, CDCl$_3$) of the alkyl glycerol ethers (s, d, t and m indicate singlet, doublet, triplet and multiplet): δ/ppm: 3.84 (1H, m, H-2), 3.70 (1H, dd, J = 3.5 Hz, J = 11 Hz, H-3a), 3.63 (1H, dd, J = 5 Hz, J = 11 Hz, H-3b), 3.50 (2H, t, J = 3.5 Hz, H-1a,1b), 3.44 (2H, dt, J = 1 Hz, J = 6 Hz, H-1′), 1.55 (2H, m, CH$_2$CH$_3$), 1.23 (s, CH$_2$), 0.85 (m, CH$_3$).

Mass spectra (GC/MS) of the nitrile derivatives of the hydrocarbon side chains: m/z [CH$_3$(CH$_2$)$_{15}$CN]: 251 (M$^+$, 1%), 236 (9%), 222 (36%), 208 (44%), 194 (31%), 180 (24%), 166 (24%), 152 (24%), 138 (35%), 124 (58%), 110 (83%), 96 (78%), 82 (56%), 68 (14%), 55 (100%); m/z [(CH$_3$)$_2$CH(CH$_2$)$_{14}$CN]: 265 (M$^+$, 1%), 250 (23%), 236 (3%), 222 (40%), 208 (19%), 194 (18%), 180 (18%), 166 (18%), 152 (21%), 138 (24%), 124 (41%), 110 (70%), 96 (55%), 82 (42%), 68 (10%), 55 (100%); m/z [CH$_3$CH$_2$CH(CH$_3$)-(CH$_2$)$_{13}$CN]: 265 (M$^+$, 1%), 250 (7%), 236 (51%), 222 (5%), 208 (15%), 194 (16%), 180 (29%), 166 (33%), 152 (32%), 138 (32%), 124 (34%), 110 (42%), 96 (40%), 82 (33%), 68 (10%), 55 (100%).

Mass spectra (GC/MS) of the pyrrolidide derivatives of the hydrocarbon side chains: m/z [CH$_3$(CH$_2$)$_{15}$CONC$_4$H$_8$]: 323 (M$^+$, 2%), 308 (1%), 294 (1%), 280 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (1%), 140 (3%), 126 (19%), 113 (100%); m/z [(CH$_3$)$_2$CH(CH$_2$)$_{14}$CONC$_4$H$_8$]: 337 (M$^+$, 2%), 322 (2%), 308 (<1%), 294 (2%), 280 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (1%), 140 (3%), 126 (20%), 113 (100%); m/z [CH$_3$CH$_2$(CH$_3$)-CH(CH$_2$)$_{13}$CONC$_4$H$_8$]: 337 (M$^+$, 2%), 322 (1%), 308 (2%), 294 (<1%), 280 (2%), 266 (<1%), 252 (<1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (2%), 140 (3%), 126 (19%), 113 (100%).

IR (CCl$_4$) of the alkyl glycerol ethers: ν_{max} = 3400, 2920, 2860, 1460, 1380, 1120, 1060.

$[\alpha]^{20}_{589}$ + 2.9° (c, 0.01 in CHCl$_3$).

Alk-1′-enyl glycerol monoethers (Fig. 2). ^{1}H NMR (300 MHz, CDCl$_3$) of the alk-1′-enyl glycerol monoethers derived after reduction of phospholipids with LiAlH$_4$: δ/ppm: 5.94 (1H, d, J = 6 Hz, H-1′), 5.39 (t, J = 5 Hz, CH=CH), 4.41 (1H, q, J = 6 Hz, J = 10 Hz, H-2′), 3.92 (1H, m, H-2), 3.80 (2H, dd, J = 2 Hz, J = 5 Hz, H-1a,1b), 3.74 (1H, q, J = 3 Hz, J = 12 Hz, H-3a), 3.66 (1H, q, J = 6 Hz, J = 12 Hz, H-3b), 2.02 (m, CH$_2$CH=CHCH$_2$), 1.27 (s, CH$_2$), 0.88 (3H, t, J = 6 Hz, CH$_3$).

Mass spectra (GC/MS) of the pyrrolidide derivatives of the hydrocarbon side chains: m/z [CH$_3$(CH$_2$)$_{14}$CONC$_4$H$_8$]: 309 (M$^+$, 1%), 294 (1%), 280 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (1%), 168 (2%), 154 (1%), 140 (3%), 126 (20%), 113 (100%); m/z [CH$_3$(CH$_2$)$_{16}$CONC$_4$H$_8$]: 337 (M$^+$, 2%), 308 (1%), 294 (1%), 266 (1%), 252 (1%), 238 (1%), 224 (1%), 210 (1%), 196 (1%), 182 (2%), 168 (3%), 154 (2%), 140 (3%), 126 (19%), 113 (100%); m/z [CH$_3$(CH$_2$)$_7$CH=CH(CH$_2$)$_7$CONC$_4$H$_8$]: 335 (M$^+$, 2%), 278 (1%), 264 (1%), 250 (1%), 236 (2%), 222 (1%), 208 (1%), 196 (1%), 182 (2%), 168 (1%), 154 (1%), 140 (3%), 126 (70%), 113 (100%); m/z [CH$_3$(CH$_2$)$_2$CH=CH(CH$_2$)$_4$CH=CH(CH$_2$)$_6$CONC$_4$H$_8$]: 333 (M$^+$, 2%), 276 (1%), 264 (1%), 250 (1%), 236 (1%), 222 (1%), 208 (1%), 194 (1%), 182 (1%), 168 (1%), 154 (1%), 140 (2%), 126 (60%), 113 (100%).

IR (CCl$_4$) of the alk-1′-enyl glycerol ethers: ν_{max} = 3400, 2920, 2860, 1660, 1460, 1120.

RESULTS AND DISCUSSION

Alkyl glycerol monoethers were isolated after flash chromatography and HPLC of total lipid extracts of *T. aurantia,* without any prior derivatization or hydrolysis

steps, and represented 2% of the total lipid and 0.02% of the sponge dry weight.

The free alkyl glycerol monoethers were identified by a combination of ^{1}H NMR, GC/MS and chemical derivatization. ^{1}H NMR experiments indicated the presence of the glycerol monoether moiety (see Fig. 1). A multiplet at 3.84 ppm assignable to the H-2 proton was coupled to two double doublets at 3.70 and 3.63 ppm that are assignable to the two H-3 protons and also to a triplet at 3.50 ppm assignable to the H-1 protons. A double triplet at 3.44 ppm assignable to the H-1′ protons was coupled to the methylene resonance at 1.55 ppm. The ^{1}H NMR spectrum was consistent with the ^{1}H NMR spectrum of commercial 1-0-hexadecylglycerol and with earlier literature data (18). The ^{1}H NMR methyl resonance was observed as a complex multiplet, which was suggestive of methyl branch points on the hydrocarbon side chain.

Mass spectroscopy (direct insertion) of the intact alkyl glycerol monoethers and GC/MS of the isopropylidene derivatives identified three species of glycerol ethers with hydrocarbon chains corresponding to 16:0 (**1**) and 17:0 (**2** and **3**). The mass spectra of the isopropylidene derivatives gave a characteristic base peak at m/z 101, which is supportive of the glycerol monoether structure (19). The mass spectra, however, did not give any information on the existence or position of any methyl branching in the hydrocarbon side chain.

To establish the position of methyl branching, the ether linkage was cleaved and the resulting alkyl iodide was derivatized to the pyrrolidide derivative for mass spectral determination of the branch position(s). N-Acylpyrrolidides have been shown to be suitable derivatives for the mass spectral localization of methyl branches and unsaturation in fatty acids (15,20). GC/MS of both the nitrile and the pyrrolidide derivatives produced diagnostic fragments indicating the presence and position of the methyl branching. The three hydrocarbon chains were identified as 17:0, 18:0 iso and 18:0 anteiso, an extra carbon atom having been added during the transformation via the nitrile derivative. Thus, the hydrocarbon side chains of compounds **1**, **2** and **3** correspond to 16:0, 17:0 iso and 17:0 anteiso, respectively (Fig. 1). The position of the methyl branch was indicated by fragments of higher intensity 14 amu above and below a low intensity fragment. The mass spectra of the nitrile derivatives, however, proved to be superior to those of the pyrrolidides, showing significantly larger intensity differences between the diagnostic fragments. For example, the 17:0 iso nitrile derivative gave fragments of mass 250 (23%), 236 (3%) and 222 (40%), while the pyrrolidide derivative showed 322 (2%), 308 (0.05%) and 294 (2%). The mass spectra of aliphatic nitrile compounds have been described previously (21).

Quantification of the individual species was achieved by GLC of the isopropylidene derivatives of compounds **1**, **2** and **3**; the results are shown in Table 1.

The specific rotation, $[\alpha]^{20}_{598}$ + 2.9°, of the alkyl glycerol monoether mixture is in numerical agreement with that found for other natural glycerol monoethers (4,22,23), all of which possess the (2S)-absolute configuration.

The reduction of phospholipids with $LiAlH_4$ or $NaAlH_2(OCH_2OCH_3)_2$ has been suggested (24) as an efficient method of isolating alkyl and alk-1′-enyl glycerol monoethers from complex lipids. In view of the presence of the unesterified glycerol monoethers in *T. aurantia*, it was of interest to examine the presence of glycerol ethers in the phospholipids of this sponge.

Reduction of *T. aurantia* phospholipids with $LiAlH_4$ allowed the isolation of both alkyl and alk-1′-enyl glycerol monoethers, which were identified by ^{1}H NMR, GC/MS and chemical derivatization. The compositions of the alkyl glycerol monoethers derived from the phospholipids were found to be identical to the unesterified alkyl glycerol monoethers. The proportion of the three species, however, was markedly different, as shown in Table 1.

The alk-1′-enyl glycerol ether moiety (see Fig. 2) was identified by ^{1}H NMR (23). A doublet at 5.94 ppm was assigned to the H-1′ proton and a quartet at 4.41 ppm to the H-2′ proton. The chemical shift and coupling constant of the H-1′ are consistent with the *cis* isomer and similar to other naturally occurring alk-1′-enyl glycerol ethers (25). The resonances between 4.0 ppm and 3.6 ppm were assignable to the protons of the glycerol moiety. Specifically, a multiplet at 3.92 ppm was assigned to the

TABLE 1

Composition of Glycerol Ethers Isolated from *T. aurantia*

Hydrocarbon side chain	Alkyl glycerol monoethers		Alk-1′-enyl glycerol monoethers
	Unesterified[a]	Phospholipids[b,c]	Phospholipids[b,d]
16:0	44	92	52
17:0 iso	32	6	—
17:0 anteiso	24	2	—
18:0	—	—	30
18:1	—	—	10
18:2	—	—	8

[a]Isolated directly without any prior chemical step. Represent 2% of the total lipids of the sponge.

[b]Isolated after reduction of phospholipids (40% of total lipids of the sponge) with $LiAlH_4$.

[c]Represent 32% of *T. aurantia* phospholipids.

[d]Represent 26% of *T. aurantia* phospholipids.

H-2 proton, the double doublets at 3.80 ppm were assigned to the H-1 protons and the two quartets at 3.74 and 3.68 ppm were assigned to the H-3 protons. Assignment of the glycerol protons was confirmed by acetylation of the mixture, which resulted in a downfield shift of ca. 1 ppm of the proton resonance(s) attached to the O-acetylated carbons. A triplet at 5.39 ppm indicated additional unsaturation in the alk-1′-enyl hydrocarbon side chain, while the methyl resonance was a clean triplet indicating no detectable methyl branching.

From the quantities of the compounds isolated, the proportions of the three species of phospholipid—diacyl, monoalkylacyl and monoalk-1′-enylacyl glycerides—were 42%, 32% and 26%, respectively. Thus, about half of the phospholipids in *T. aurantia*, which represent 40% of the total lipids extracted, contain an ether linkage.

To establish the identities of the hydrocarbon side chains of the alk-1′-enyl glycerol ethers, the ether bond was hydrolyzed with HCl to yield aldehydes and glycerol (16). The aldehydes were then oxidized to the corresponding acids and subsequently transformed into pyrrolidides. GC/MS of the pyrrolidide derivatives and GLC of the methyl esters enabled identification and quantification of the hydrocarbon side chains as 16:0, 18:0, 18:1 and 18:2 (see Fig. 2 and Table 1). The positions of unsaturation were identified by GC/MS by an interval of 12 amu in the fragmentation instead of the regular 14 amu spacing and suggested the unsaturation positions to be Δ^{9}-18:1 and $\Delta^{8,14}$-18:2. For example, Δ^{9}-18:1 showed characteristic fragments at m/z 222, 208, 196 and 182, while the $\Delta^{8,14}$-18:2 acid displayed characteristic fragments at m/z 276, 264 and 250 and at 208, 194, 182 and 168. The suggested unsaturation positions at $\Delta^{8,14}$-18:2 are to our knowledge previously unknown. Insufficient material was available to provide more secure evidence for the location of these double bonds.

Incorporation experiments were carried out with *T. aurantia* using both intact sponges and dissociated sponge cells. Different incorporation results were obtained using [1-^{14}C]hexadecanol with the two systems. Significant incorporation (1.1% of the total activity added) into the unesterified alkyl glycerol monoethers was obtained with dissociated sponge cells, whereas the esterified (i.e., phospholipid-derived) alkyl and alk-1′-enyl glycerol ethers contained essentially no radioactivity (<0.03% of the total activity added).

The incorporation experiments with intact sponges resulted primarily in the oxidation of the [1-^{14}C]hexadecanol to the acid and incorporation of the acid into the phospholipids; thus 1.7% and 1.1% of the total activity added was recovered, after 7 and 17 days, respectively, as fatty alcohols after reduction of the phospholipids. The incorporation of fatty alcohols primarily into complex lipids after being oxidized has also been observed for various mammalian tissues (26). The glycerol ether fractions contained essentially no radioactivity (<0.08% of the total activity added).

Screening a number of marine sponges of the class Demospongiae—*Aplysina fistularis* (Order Verongida), *Microciona prolifera* (O. Poecibsclerida), *Pseudaxinyssa* sp., Australian Museum Specimen #Z4988 (O. Axinellida) and *Reneira* sp. (O. Haplosclerida)—did not uncover any detectable level of unesterified alkyl glycerol monoethers, thus showing that this type of ether lipid is not widely distributed among sponges. No evidence of any di- or triglycerides of any form was obtained.

The unesterified alkyl glycerol monoethers 2 and 3, isolated from *T. aurantia*, are novel compounds. The ether 1, although previously reported, so far has not been encountered in unesterified form. Their biosynthesis can be explained on the basis of known enzymatic pathways (27) and represents a branch of the usual biosynthetic pathway to esterified glycerol ethers. The hydrocarbon side chains of the alkyl glycerol ethers, especially the 17:0 iso and anteiso chains, and possibly 16:0, are probably of bacterial origin, being either assimilated from dietary sources, which consist largely of bacteria (28), and/or contributed from the presumably symbiotic microorganisms residing in the sponge matrix. (*T. aurantia* contains numerous microorganisms as shown by electron microscopy; unpublished results.) Recently it has been shown that short-chain straight and branched fatty acids are elongated and desaturated by the sponges *Jaspis stellifera* (29) and *Aplysina fistularis* (30). The ^{14}C incorporation experiment with dissociated sponge cells suggests that the unesterified alkyl glycerol monoethers are, however, biosynthesized by *T. aurantia* and are not of dietary origin. The difference between the hydrocarbon side chain composition of the esterified alkyl and alk-1′-enyl glycerol monoethers (see Table 1) is surprising in view of the fact that alkyl glycerol ethers are considered (26) to be the precursors of the alk-1′-enyl glycerol ethers.

Alkyl glycerol monoethers have been found to possess potent antimicrobial activity, affecting both glycerolipid and lipoteichoic acid biosynthesis in *Streptococcus mutans* (31). Thus, it is possible that the alkyl glycerol monoethers isolated from *T. aurantia* may play a defensive role as antimicrobial agents.

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Influence of Environmental Medium on Fatty Acid Composition of Human Cells: Leukocytes and Fibroblasts

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Fibroblasts in culture and leukocytes have been widely used to study fatty acid and lipoprotein cellular metabolism. The present investigations were designed to study the role of nutritional and environmental factors on lipid metabolism in these two types of cells. Leukocytes freshly isolated from human blood and fibroblasts cultured in media enriched in human serum (HS) have relatively similar fatty acid distributions. However, more important differences are observed in fibroblasts cultured in media enriched with HS or with fetal bovine serum (FBS). It is obvious that the quantity and quality of fatty acids are very different in FBS and HS, but intracellular regulation ensures relative homogeneity of saturated (SFA) and monounsaturated fatty acids (MUFA) in the cells, particularly in phospholipids.

The first modifications induced by different media (FBS or HS) are detected on cellular growth; the differences seem to be due more to the fatty acid (FA) quantitative supply than to the FA quality of each culture medium. The major modifications in FA composition induced by different culture media concern the polyunsaturated fatty acids (PUFA) of phospholipids, especially the n-6 family. The intracellular linoleic acid level depends on the level in the medium, but intracellular n-6 metabolite levels depend both on the level in the medium and on the growth state of the cells. The n-3 family seems to be less affected by the quality of the medium in our experiment, and the cells maintain a stable docosahexaenoic acid (22:6n-3) level. A higher content of the n-3 family in the medium induces a higher level of eicosa- or docosapentaenoic acid, rather than docosahexaenoic acid itself.

Finally, the FA quality of the medium influences the cellular PUFA content but, with a low FA quantitative supply, the FA quality of the medium has less influence on the cellular PUFA quality, and apparently has no effect on the SFA content of phospholipids. Modification of the quantitative supply of the medium and of the quality of the cells (strain and growing state) are more important for the distribution of SFA and MUFA in the neutral lipids of the cells.
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Fibroblasts in culture and leukocytes have been widely used to study the cellular metabolism of fatty acids and lipoproteins. Recently, such systems have furthered the understanding of the mechanism of dyslipoproteinemias and diseases induced by lipid metabolism disturbances (1-3).

Cell cultures have provided a good system for studying lipid metabolism; indeed, in vivo studies on whole animals are often too complex or give conflicting results. However, in vitro cell studies are infrequently used to evaluate the role of dyslipidemic situations or various nutritional states on the cellular fatty acid metabolism (4,5).

Two major problems were encountered using cell cultures to study fatty acid (FA) metabolism: First, by comparing the results obtained in other laboratories, it became evident that single, minor modifications in the quality and the quantity of the medium can induce major differences in the results. For example, endothelial cells only express Δ5 and Δ6 FA desaturase activities in a poorly enriched serum medium (5,6). Second, because many experiments performed with massive amounts of a single free fatty acid (FFA) added to the medium have resulted in accumulation of toxic phenomena, which would probably never be encountered in whole organisms (7,8), it seems important to reproduce these physiological conditions as closely as possible. Valuable information can be gained from studies on the possible FA modifications obtained with culture media resembling human serum or interstitial fluid.

Another suitable tissue for lipid metabolic analysis is the leukocyte, because of its accessibility. It has been found to reflect a wide range of metabolic disturbances observed in numerous diseases affecting carbohydrate and lipid metabolism (1,9). In comparison with in vitro cultured cells, leukocytes, like fibroblasts, could be considered to represent an ex vivo steady state.

The present study was designed to examine whether, by using intact leukocytes as experimental tissue, it was possible to demonstrate the in vivo state of cellular FA composition, and whether this model could be related to the FA composition of fibroblasts cultured in vitro with human serum (HS)-enriched medium, instead of the fetal bovine serum (FBS)-enriched medium conventionally used. The importance of nutritional conditions in relation to cellular type and culture conditions on cellular FA composition is discussed.

MATERIALS AND METHODS

Materials. Human blood samples, leukocytes and skin fibroblasts were obtained from healthy female and male normolipemic subjects between 25 and 35 years of age, with total cholesterol between 180 and 220 mg/dl and triglycerides between 80 and 150 mg/dl. For culture medium, FBS was purchased from Eurobio (Paris, France); for in vitro isolation of leukocytes, Ficoll-paque was obtained from Pharmacia (Bois d'Arcy, France). The different FA used as standards for gas chromatography (GC) were obtained from Sigma (St. Louis, Missouri) and NuChek Prep (Elysian, Minnesota).

Cell cultures. Stock cultures of fibroblasts were maintained in 75 cm^2 flasks containing 30 ml of RPMI 16/40 supplemented with penicillin (100 μg/ml), streptomycin (100 μg/ml), Fungizone (5 μg/ml), 1% (v/v) L-glutamine (200 mM) and 10% (v/v) FBS.

Cells were maintained at 37 C in a humidified atmosphere of air/CO_2 (95:5). For all experiments, cells between five and 13 passages and 7-day cultures in 25 cm^2 flasks containing 10 ml of RPMI generally supplemented with 10% FBS or 10% HS were used.

A single batch of FBS was used in all experiments, and seven different human sera were tested. The FA

percentage compositions of FBS and HS were established (Table 1), as well as estimations of FA quantities (μg/ml medium) to determine the relative FA values of 10% FBS and HS culture media. When modified serum concentrations and culture durations were tested, this was indicated in the text and the tables.

Leukocyte preparation. Blood obtained by venipuncture (35 ml) was anticoagulated with heparin from fasting healthy donors (four female and one male), and leukocytes were isolated using the classical Ficoll-paque isolation procedure (10). Leukocyte preparations were washed three times with Puck EDTA solution (8 g NaCl, 0.4 G KCl, 1 g glucose, 0.35 g $NaHCO_3$, and 0.2 g EDTA per liter). Each experiment was performed on 3×10^6 cells (the smallest number of cells permitting FA analysis).

GC in glass capillary column. Methyl esters of FA from total lipids, phospholipids (PL), FFA, triglycerides (TG) and cholesteryl esters (CE) of each fraction were treated with methanol/benzene/sulfuric acid (100:0.2:2, v/v/v) for 3 hr at 80 C. The methyl esters were agitated for 1 min with a mixture of heptane/water (v/v), and the heptane phase was removed and evaporated.

FA analysis was performed on a Hewlett-Packard gas chromatograph 5880 A fitted with a hydrogen flame ionization detector and a 25-m glass column of butane diol-succinate (BDS) provided by the Society Lesieur-Cotelle. The oven was programmed from an initial phase of 4 min at 160–190 C for 17 min with a progression rate of 2 C/min. The peaks were identified by comparison of the retention times with those of known standards. Quantitation was performed using heptadodecanoic acid (17:0) as an internal standard introduced before transmethylation, and relative quantities of individual FA were expressed as a percentage of the total by comparison of peak areas using a Hewlett-Packard 3380 A integrator.

FA extraction and quantitation. Monolayer cell cultures were washed three times with Dulbecco phosphate buffered saline (pH 7.2) and covered for several minutes at

TABLE 1

Fatty Acid Composition of the Culture Media[a]

Fatty acids	FBS (%) (n = 3)	HS (%) (n = 7)	μg FA/ml medium 10% enriched in FBS (n = 3)	μg FA/ml medium 10% enriched in HS (n = 7)
Total FA %	100	100		
μg FA/mg proteins			1.4	3.2
Total SFA	42.58	31.86	23.48	77.81
14:0	2.85 ± 1.25	1.15 ± 0.36	1.57	2.81
16:0	23.84 ± 1.61	21.03 ± 1.53	13.15	51.36
18:0	13.67 ± 1.78	8.11 ± 0.96	7.54	19.81
20:0	0.66 ± 0.16	0.28 ± 0.08	0.36	0.68
22:0	0.97 ± 0.26	0.71 ± 0.12	0.54	1.73
24:0	0.59 ± 0.26	0.58 ± 0.08	0.32	1.42
Total MUFA	32.11	21.53	17.70	52.92
16:1	6.12 ± 0.35	2.36 ± 0.65	3.37	5.76
18:1	24.01 ± 2.67	18.11 ± 2.38	13.24	44.23
20:1	0.40 ± 0.13	0.24 ± 0.08	0.22	0.59
24:1	1.58 ± 0.43	0.82 ± 0.08	0.87	2.34
Total PUFA	22.47	45.89	12.40	112.11
Total n-6	14.79	42.07	8.16	102.78
18:2n-6 met	10.01	9.97	5.52	24.38
18:3n-6 met	8.12	9.07	5.52	23.89
18:2	4.78 ± 1.03	32.10 ± 3.83	2.64	78.40
18:3	1.89 ± 1.44	0.70 ± 0.16	1.04	1.71
20:2	—	0.20 ± 0.05	—	0.49
20:3	1.47 ± 0.09	1.26 ± 0.27	0.81	3.08
20:4	6.49 ± 1.15	7.53 ± 0.82	3.58	18.39
22:4	0.16 ± 0.06	0.14 ± 0.07	0.09	0.34
22:5	—	0.14 ± 0.03	—	0.37
Total n-3	7.68	3.82	4.24	9.33
18:3n-3 met	7.14	3.41	3.94	8.33
18:3	0.54 ± 0.34	0.41 ± 0.10	0.30	1.00
20:5	1.23 ± 0.32	0.70 ± 0.32	0.68	1.71
22:5	2.29 ± 0.13	0.46 ± 0.03	1.26	1.12
22:6	3.62 ± 0.43	2.25 ± 0.53	2.00	5.50
n-6/n-3	1.93	11.01		
18:2n-6 met/18:3n-3 met	1.40	2.92		
18:3n-6 met/18:3n-3 met	1.14	2.66		

[a]FA, fatty acid; FBS, fetal bovine serum; HS, human serum; SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; met, metabolites. n, Number of serum tested or number of analyses of the same lot of fibroblasts. Each experiment was done in triplicate. Total FA were quantified using heptadecanoic acid (17:0) as a standard.

37 C by a trypsin film (0.05%) in Puck EDTA solution until detachment of the cells occurred. The fibroblasts were then isolated from the suspension by sedimentation at 1200 G for 5 min, and the pellet was resuspended in 2 ml of distilled water before being disrupted by seven freeze-thaw cycles; 0.5 ml was removed for protein determination by the method of Lowry et al. (11), and 2.7 ml of chloroform/methanol (2:1) was added to the remaining preparation (1.5 ml) for lipid extraction by the procedure of Folch et al. (12). Two other extractions of the aqueous phase were performed under the same conditions, and the pooled organic phases were evaporated to dryness under a stream of nitrogen.

Bursting and extraction of leukocytes were performed in the same manner from a suspension of 3×10^6 cells/ml of distilled water.

For thin layer chromatography, the extracted material was resuspended in 50 μl of chloroform and spotted on methanol prewashed Silica Gel G plates (250 μm thick; 1505/LS 254 from Schleicher-Schul), and then developed in petroleum ether/diethyl ether/acetic acid (90:30:1, v/v/v). Lipid spots were visualized by vaporization of a rhodamine 6G solution in ethanol (0.05%) and identified by comparison with known standards. Each lipid class was scraped off with silica gel before transmethylation.

Results were expressed as percentages to evaluate the variations in the FA distribution and in nanograms of FA per unit of cellular protein (μg) to appreciate the absolute quantitative differences or similarities in the cell FA for each type of culture. The amount of protein in each flask was closely related to the number of cells, but there were different coefficients for different cell types (leukocytes or fibroblasts). Analyses were performed in triplicate on 3×10^6 leukocytes or 25 cm^3 flask culture for fibroblasts and on 50 μl of HS and FBS.

RESULTS

FA composition of fibroblasts cultured in HS-enriched medium and of freshly isolated human leukocytes. The FA composition of freshly isolated leukocytes (expressed in percentage of total FA) was quite similar to the composition of fibroblasts cultured for seven days in 10% HS-enriched medium. Analysis of the two essential PUFA families, n-6 and n-3, showed some minor quantitative variations in these two cell types. The higher levels of n-6 FA in leukocytes compared to fibroblasts seems to be due to 18:2 FA metabolites (essentially arachidonic acid) more than to linoleic acid itself, unlike the n-3 family, which showed a decrease in the last metabolite (22:6n-3 docosahexaenoic acid).

These differences were demonstrated by calculating the n-6/n-3 ratio and 18:2n-6 metabolites/18:3n-3 metabolites ratio, which were increased in leukocytes compared to fibroblasts cultured in HS. No other major differences in FFA, TG, CE or PL were observed between leukocytes and fibroblasts cultured in HS (Tables 2 and 3). However, the arachidonic acid level was slightly higher in leukocyte neutral lipids than in fibroblasts.

FA composition of fibroblasts cultured in FBS or HS (Tables 2 and 3). Cells cultured over the same time period in a medium enriched with 10% HS or 10% FBS showed differences in polyunsaturated fatty acids (PUFA). Cells cultured in FBS showed a considerable reduction of PUFA; the most marked differences were in the n-6 family with a decrease of 50% and a corresponding increase of 30% in the n-3 family (the difference being more important for 22:5n-3 than for 22:6n-3). However, each member of the n-6 family was not altered to the same extent, and the discrepancies observed for each metabolite of 18:2n-6 were not as important as for 18:2 itself, which was three times less ($p < 0.01$) in FBS-cultured fibroblasts. On the other hand, 18:3n-6 derived from Δ6-desaturation of 18:2n-6 was increased by a factor of 2.5. The next link of this metabolic chain, 20:3n-6, was equally represented, but arachidonic acid (20:4n-6) was also reduced in FBS-cultured fibroblasts, together with 22:4n-6 ($p < 0.05$). The n-6/n-3 ratio reflected these differences, as the n-6/n-3 ratio was twice as high in cells cultured in HS than in FBS-cultured cells.

Although the distribution of FA differed to a certain extent in FBS- and HS-cultured fibroblasts, it must be

TABLE 2

Fatty Acid Composition of Freshly Isolated Human Leukocytes and Cultured Fibroblasts with FBS and HS 10% Enriched Media[a]

	Fibroblasts		
Fatty acids (%)	FBS (n=6)	HS (n=7)	Leukocytes (n=5)
SFA			
14:0	2.20±0.49	1.22±0.53	1.73±0.38
16:0	20.83±1.71	21.64±0.88	20.78±2.36
18:0	9.97±3.57	17.38±2.90	20.64±0.93
20:0	0.81±0.36	0.40±0.15	0.80±0.14
22:0	1.08±0.20	0.82±0.15	1.45±0.34
24:0	1.52±0.42	1.14±0.25	0.97±0.13
MUFA			
16:1	4.50±1.03	2.94±1.04	2.64±0.78
18:1	19.65±4.46	18.52±1.70	13.00±0.41
20:1	0.61±0.20	0.39±0.10	1.70±1.18
22:1	0.37±0.06	0.25±0.10	0.53±0.04
24:1	1.16±0.40	1.19±0.12	0.87±0.29
PUFA			
n-6 family	13.50	27.02	29.51
18:2n-6 met	10.74	17.49	21.34
18:3n-6 met	8.37	15.76	18.97
18:2n-6	2.76±1.02	9.53±1.87	8.17±1.82
18:3	2.37±0.79	1.05±0.34	1.87±1.06
20:2	—	0.68±0.28	0.50±0.14
20:3	0.81±0.23	0.92±0.14	2.62±0.80
20:4	6.15±2.87	11.18±1.02	14.78±2.58
22:4	1.41±0.57	3.66±0.57	1.57±0.41
n-3 family	6.80	4.61	4.39
18:3n-3 met	6.44	4.46	3.41
18:3n-3	0.36±0.20	0.15±0.09	0.98±0.60
20:5	0.96±0.49	0.20±0.03	0.30±0.15
22:5	2.34±1.17	1.42±0.38	1.40±0.38
22:6	3.14±0.75	2.84±0.49	1.71±0.42
n-6/n-3	1.99	5.86	6.72
18:2n-6 met/18:3n-3 met	1.67	3.92	6.26
18:3n-6 met/18:3n-3 met	1.30	3.53	5.56
μg FA/protein unit (μg)	273	258	137

[a]FBS, fetal bovine serum; HS, human serum; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; met, metabolites. n, Number of determinations. Each experiment was performed in triplicate. Total FA were quantified using heptodecanoic acid (17:0) as a standard. Since some of the FA were positively identified, the values for FA classes do not add up to 100%.

TABLE 3

Fatty Acid Distribution in Lipid Classes of Freshly Isolated Human Leukocytes and Cultured Fibroblasts with FBS and HS 10% Enriched Media

	Phospholipid FA			Free FA			Triglyceride FA			Cholesteryl esters FA		
	Fibroblasts			Fibroblasts			Fibroblasts			Fibroblasts		
Fatty acids (%)	FBS (4)[a]	HS (6)	Leukocytes (3)	FBS (4)	HS (6)	Leukocytes (5)	FBS (4)	HS (6)	Leukocytes (5)	FBS (4)	HS (6)	Leukocytes (5)
Total SFA	52.63	46.99	48.99	77.30	75.64	76.80	64.00	70.30	68.56	71.80	75.70	64.31
14:0	3.37	0.79	0.96	8.88	8.29	12.11	9.33	10.23	12.75	25.44	33.22	12.16
16:0	25.61	23.52	20.45	39.70	40.90	36.07	37.50	39.36	35.92	28.96	27.80	33.93
18:0	19.56	19.84	23.95	25.81	24.59	25.59	15.08	18.73	16.10	15.19	12.93	14.60
20:0	0.77	0.46	0.77	0.80	0.80	1.11	0.91	0.82	1.10	0.92	1.02	1.77
22:0	1.72	1.02	1.61	1.11	1.06	0.93	1.18	1.16	1.03	1.29	0.73	1.85
24:0	1.60	1.36	1.25	1.00	—	0.99	—	—	1.66	—	—	—
Total MUFA	26.90	23.08	17.75	13.40	13.49	11.35	22.86	17.98	15.73	14.24	11.85	16.06
16:1	5.60	2.62	1.64	4.72	4.35	4.09	7.68	6.77	5.72	5.62	5.20	7.13
18:1	20.74	19.27	13.93	7.62	8.60	4.62	13.11	9.66	7.15	6.16	5.08	7.22
20:1	0.55	0.36	0.46	1.06	0.54	1.49	2.07	1.55	1.39	1.90	1.36	1.71
22:1	—	—	—	—	—	1.15	—	—	1.47	0.56	0.21	—
24:1	—	0.83	1.72	—	—	—	—	—	—	—	—	—
Total PUFA	20.28	29.92	33.23	9.32	10.49	11.55	12.71	11.70	15.17	13.19	11.69	18.81
Total n-6 family	12.90	24.80	28.21	7.29	9.34	7.19	9.17	8.76	10.36	8.37	6.98	11.23
18:2n-6 met	8.11	15.76	20.80	3.08	5.19	4.40	4.43	3.75	5.61	4.26	3.93	7.63
18:3n-6 met	6.58	15.03	20.19	1.19	2.72	1.97	0.60	0.62	2.06	0.81	0.85	3.02
18:2	4.79	9.04	7.41	4.21	4.15	2.79	4.74	5.01	4.75	4.11	3.05	3.60
18:3	1.53	0.73	0.61	1.89	2.47	2.43	3.83	3.13	3.55	3.45	3.08	4.61
20:2	—	0.75	0.56	—	—	—	—	—	—	—	—	—
20:3	0.96	0.91	1.20	0.44	0.42	0.39	0.60	0.62	0.55	0.81	0.85	1.32
20:4	4.28	10.25	16.52	0.75	1.66	1.58	—	—	1.51	—	—	1.70
22:4	1.33	3.12	1.91	—	0.64	—	—	—	—	—	—	—
22:5	—	—	—	—	—	—	—	—	—	—	—	—
Total n-3 family	7.38	5.10	5.02	2.03	1.15	4.36	3.54	2.94	4.81	4.83	4.71	7.58
18:3n-3 met	6.84	4.74	3.86	—	—	1.97	0.26	—	0.65	0.35	0.35	0.91
18:3	0.54	0.36	1.16	2.03	1.50	2.39	3.28	2.94	4.16	4.48	4.36	6.67
20:5	0.84	0.23	0.41	—	—	0.43	0.26	—	0.65	0.35	0.35	0.91
22:5	2.93	1.66	1.33	—	—	—	—	—	—	—	—	—
22:6	3.06	2.85	2.12	—	—	1.54	—	—	—	—	—	—
n-6/n-3	1.75	4.86	5.75	3.59	9.12	1.65	2.59	2.98	2.15	1.73	1.48	1.48
18:2n-6 met/18:2n-3 met	1.19	3.32	5.39	—	—	2.23	—	—	—	—	—	—
18:3n-6 met/18:3n-3 met	0.96	3.17	5.23	—	—	1.00	—	—	—	—	—	—

FA, fatty acid; FBS, fetal bovine serum; HS, human serum; SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; met, metabolites.

[a](n), Number of determinations.

noted that the cell growth was also different after seven days of culture, being half with FBS compared to HS cultures (estimated by the procedure of Lowry et al.). Lower growth was generally accompanied by a small increase in the ratio of total FA per unit of cellular protein related to the FA triglyceride content (40 to 75% more).

Analysis of the FA pattern in lipid classes (PL, TG, CE, FFA) of the two forms of cultured fibroblasts (Table 3) showed no striking differences in neutral lipids, which were essentially 90% saturated or monounsaturated chains.

FA pattern of culture medium and fibroblast FA composition. The FA compositions of HS and FBS were studied to explain differences observed in the FA composition of fibroblasts cultured in media enriched by different additives.

Table 1 shows the distribution of FA expressed as a percentage and also an estimation of FA quantities provided to the cells at the beginning of the culture (in μg FA/ml of medium enriched with 10% FBS or HS). It is obvious that the quality and quantity of FA is very different in FBS and HS. Qualitatively HS is poorer in saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) and richer in PUFA, but the distribution of n-6 and n-3 families is quite different, i.e. 18:2n-6 is well represented in HS and 18:2n-6 metabolites are equally represented. The n-3 family in HS is one-half of that of FBS, which results in a very different n-6/n-3, but the n-6/n-3 metabolite ratio does not differ to the same extent.

Moreover, quantitatively, it must be noted that FBS has one-half the protein content of HS and 4–5 times lower total FA content. Apart from differences due to the distribution of FA, this results in different contributions of culture media usually enriched with 10% HS or FBS. When 10% HS is used instead of 10% FBS, the cultured cells bathe in a medium containing 30 times more 18:2n-6

and four times more 18:2n-6 metabolites (with apparently the same percentage in FBS and HS) and twice as much n-3 (with apparently half the quantity expressed in %).

Medium sera concentrations and FA pattern of fibroblasts. As described above, culture in 10% FBS or HS does not provide the same FA qualitative and quantitative supply to cells. To determine how the serum FA level introduced into the culture medium can modify the quality of cellular FA, we added FBS or HS at different concentrations to the medium, in order to give approximately iso FA quantitative supply to the cells with each kind of serum (20% FBS and 5% HS, or 10% FBS and 2.5% HS) (Table 4).

Results show that the cellular PUFA increased with the HS content of the medium and remained stable with the FBS medium. Moreover, a global enrichment of the medium with the same serum, without changing the quality of FA (unchanged n-6/n-3 ratio), induces a selective modification of the cellular PUFA quality. HS-enriched culture showed more n-6 metabolites (+50%), more 18:2n-6 (+20%) and a blockade of Δ6 (−75% 18:3n-6), but minor modifications in the n-3. In FBS-cultured cells we observed similar findings, but to a lesser degree. These modifications in PUFA of total FA are corroborated by the analysis of cellular phospholipids, which showed minor modifications of SFA or MUFA. Finally, an identical FA quantitative supply, even with different FA quality for medium enrichment (using FBS or HS), reduces the differences in PUFA quality of cultured cells, especially with low quantitative supply (compare FBS 20% and HS 5% or FBS 10% and HS 2.5%). Differences in PUFA result mainly in a greater cellular enrichment of 18:2n-6, related to its concentration in the medium.

It must be noted that cell growth is related to FA quantitative supply, independent of the serum type (compare iso FA supply, FBS 10% and HS 2.5%). Doubling each serum concentration in the medium induces a 60–100% increase in the quantity of cellular protein for a 7-day culture. Reciprocally, the level of fatty acids per unit of protein was reduced, and this was linked to a loss of neutral lipids (essentially triglycerides in FBS cultured cells), leading to a reduction in SFA and MUFA. Modifications were not as clear or as systematically observed in FBS as in HS cultured cells.

Cellular growth and FA pattern of fibroblasts cultured in FBS and HS (Table 5). To verify whether the differences previously observed were simply related to growth state, the FA compositions of fibroblasts at different culture times were compared at days 4 and 6 in 10% FBS or HS. For the cells obtained in FBS, due to the lower supply contribution of the medium, growth was already stabilized at day 4 (measured by the protein content); for cells cultured in HS, the growth increased by 75% between day 4 and day 6 and then stabilized. Here also, the growth appeared related to a decrease in SFA and MUFA (20% in the cells cultured in HS).

We noticed that, using these two different media, only minor differences in n-6 and n-3 metabolites were found, with more enrichment in n-3 for FBS cultures (75%) and in n-6 for HS cultures (+33% for 20:4n-6); major differences in cellular PUFA were still due to the 18:2n-6 levels related to the composition of the medium.

Stability of cells cultured successively in different media. After seven days of culture, the medium was removed and cells were refed with the same type of medium (HS or FBS) and were harvested five days later. We observed that cellular protein did not change, but that the cells continued to be enriched in n-6 metabolites, essentially in 20:4n-6, without appreciable differences in the n-3 family.

In another experiment, after seven days of culture in 10% FBS or HS, the medium was changed with replacement of FBS by HS medium and vice versa. Five days later, FA modifications were almost complete for FBS

TABLE 4

Fatty Acid Analysis of Fibroblasts Cultured in Media Enriched with Different Concentrations of FBS and HS

	FBS (μg FA/μg proteins)		HS (μg FA/μg proteins)		
	10% (n = 6)	20% (n = 6)	2.5% (n = 3)	5% (n = 3)	10% (n = 3)
Cellular proteins (μg/flask)	90 ± 4	150 ± 1	88 ± 2	132 ± 8	190 ± 6
Total FA	341 ± 30	274 ± 14	455 ± 20	336 ± 7	287 ± 7
SFA	198 ± 18	146 ± 11	245 ± 16	162 ± 5	119 ± 3
MUFA	77 ± 8	63 ± 1	117 ± 15	82 ± 1	71 ± 2
PUFA	65 ± 5	65 ± 3	83 ± 3	91 ± 2	97 ± 1
Total n-6 family	45 ± 5	47 ± 4	69 ± 3	74 ± 2	80 ± 1
18:2n-6	7.5 ± 2.0	6.0 ± 0.7	26.5 ± 2.9	29.9 ± 2.1	30.9 ± 0.7
18:3	11.8 ± 3.1	11.0 ± 4.2	15.2 ± 2.3	6.4 ± 2.1	4.3 ± 1.0
20:2	—	—	—	1.8 ± 0.1	2.3 ± 0.1
20:3	2.2 ± 0.2	2.1 ± 0.2	2.2 ± 0.1	3.1 ± 0.1	3.1 ± 0.1
20:4	19.9 ± 0.1	22.9 ± 0.9	20.5 ± 2.1	25.6 ± 1.8	29.9 ± 0.7
22:4	3.5 ± 0.2	5.1 ± 0.5	4.1 ± 0.5	6.3 ± 0.3	8.7 ± 0.1
18:3n-3 family met	19 ± 1	16.8 ± 0.6	10 ± 0.5	15 ± 1	16 ± 0.6
20:5	2.7 ± 0.3	1.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
22:5	6.6 ± 0.9	5.8 ± 0.5	2.1 ± 0.1	4.1 ± 0.3	4.2 ± 0.2
22:6	9.3 ± 0.1	8.5 ± 0.5	7.4 ± 0.4	10.1 ± 0.8	11.2 ± 0.3

FBS, fetal bovine serum; HS, human serum; FA, fatty acid; SFA, saturated FA; MUFA, monounsaturated FA; PUFA, polyunsaturated FA; met, metabolites.

TABLE 5

Cellular Growth and Fatty Acid Analysis of Fibroblasts

	FBS 10%		HS 10%	
	D_4 (n = 3)	D_6 (n = 4)	D_4 (n = 3)	D_6 (n = 6)
Cellular proteins				
μg/Flask	90 ± 2	98 ± 2	132 ± 21	208 ± 7
μg FA/μg proteins	269 ± 11	275 ± 3	300 ± 35	243 ± 45
Saturated FA	132 ± 3	117 ± 3	147 ± 28	110 ± 12
Monounsaturated FA	77 ± 5	84 ± 2	75 ± 10	60 ± 5
Polyunsaturated FA	60 ± 3	73 ± 7	78 ± 10	74 ± 7
Total n-6 FA family	46.4 ± 2.3	51.3 ± 4.5	66.9 ± 7.8	65.2 ± 5.8
18:2	5.4 ± 0.1	6.4 ± 1.1	16.3 ± 1.2	15.7 ± 1.7
18:3	6.1 ± 0.9	9.2 ± 3	12.1 ± 1.3	3.6 ± 0.6
20:3	1.8 ± 0.1	1.8 ± 0.1	2.0 ± 0.2	2.2 ± 0.2
20:4	27.2 ± 1.2	29.5 ± 0.6	25.7 ± 4.9	34.5 ± 2.6
22:4	5.9 ± 0.2	4.4 ± 0.3	9.6 ± 1.1	8.5 ± 0.7
n-3 FA met	11.5 ± 1.4	18.6 ± 0.5	8.7 ± 2.3	9.1 ± 0.4
20:5	1.5 ± 0.1	3.6 ± 0.3	—	—
22:5	3.9 ± 0.2	6.2 ± 0.4	3.1 ± 1.0	2.9 ± 0.1
22:6	6.1 ± 0.2	8.8 ± 0.2	5.5 ± 1.0	6.2 ± 0.4

FBS, fetal bovine serum; HS, human serum; D_4, 4 days' culture time; D_6, 6 days' culture time; FA, fatty acid; met, metabolites.

TABLE 6

Fatty Acid Analysis of Fibroblasts Cultured Successively in Different Media

	Culture medium[a]			
	A (n = 3)	B (n = 3)	C (n = 3)	D (n = 3)
Cellular growth				
(μg protein/flask)	147 ± 2	495 ± 7	500 ± 10	590 ± 10
FA (μg/μg Protein)				
n-6 family	27	49.9	62.1	65.0
18:2	4.5 ± 0.5	12.5 ± 0.9	19.6 ± 2.0	22.6 ± 0.7
20:4	16.4 ± 0.9	28.7 ± 1.0	30.2 ± 0.7	30.1 ± 0.7
22:4	4.3 ± 0.5	6.2 ± 0.5	8.3 ± 0.5	9.6 ± 0.5
18:3n-3 met	17.0	15.5	12.7	9.6
20:5	1.7 ± 0.2	1.0 ± 0.1	0.3 ± 0.1	—
22:5	8.3 ± 0.6	6.2 ± 0.5	5.2 ± 0.3	3.0 ± 0.2
22:6	7.0 ± 0.5	8.2 ± 0.8	7.2 ± 0.8	6.6 ± 0.4

[a]A, 7 days fetal bovine serum (FBS); B, 7 days human serum (HS) + 5 days FBS; C, 7 days FBS + 5 days HS; D, 7 days HS. FA, fatty acid; met, metabolites. After a seven-day culture in 10% FBS (A) or HS (B), the medium is changed with inversion of FBS for HS culture (C) and vice versa (B). FA analysis is compared to normal culture in HS (D) or FBS (A).

culture secondarily bathing in HS (Table 6; A,C,D) except for 22:5n-3, which did not reach values generally obtained in cultures in HS (about one-third of its initial level in FBS). Modifications obtained in cells first cultured in HS and then in FBS were not as complete, and intermediary levels of FA were generally obtained when compared to cells cultured for seven days in FBS (Table 6; D,B,A). With each culture type, for the n-3 family, there were no modifications in the last metabolite 22:6n-3.

Influence of fibroblast strains and individual HS on FA distribution in fibroblasts. In all experiments, apart from the fibroblast strains, certain variations were observed in the distribution of FA in different cellular lipid classes, showing higher levels of cellular neutral lipids with a lower cellular protein content in the 7-day culture (Table 7).

As expected, the use of individual control HS with different FA composition for culture medium led to different distributions of cellular FA, especially of PUFA. For example (Table 8), two control sera differing only in some n-6 and n-3 fatty acids were used to culture the same fibroblast strain in 10% HS-enriched medium. The cellular level of each FA was related to its level in the serum used. However, the cellular 22:6n-3 was more stable regardless

TABLE 7

Influence of Different Control Fibroblast Strains on Cellular Growth and Fatty Acid Distribution in Lipid Classes

	Fetal bovine serum		Human serum		
	Strain D (n = 4)	Strain R (n = 2)	Strain D (n = 6)	Strain R (n = 5)	Strain L (n = 4)
Cellular growth (μg protein/flask)	63 ± 10	146 ± 20	320 ± 17	464 ± 37	400 ± 30
Fatty acid distribution in lipid classes (%)					
Phospholipids	30 ± 4	77 ± 4	49 ± 5	80 ± 4	72 ± 4
Neutral lipids	70 ± 4	23 ± 4	51 ± 5	20 ± 4	28 ± 4

TABLE 8

Influence of PUFA Quality of Human Serum-Enriched Medium on PUFA Distribution in Cultured Fibroblasts

	Control serum[a]			Cultured fibroblasts in human serum		
Fatty acid	A (%)	B (%)	B vs A (%)	A	B	B vs A (%)
				(μg FA/μg cellular protein)		
n-6 Family						
18:2	35.2	40.4	−14	26.4 ± 2.4	22.6 ± 0.7	−14
20:4	6.0	9.7	+61	18.1 ± 2.0	30.1 ± 0.7	+66
22:4	0.2	0.3		5.4 ± 1.1	9.6 ± 0.1	+77
n-3 Family						
20:5	0.6	0.3	−50	—	—	
22:5	0.6	0.3	−50	4.2 ± 0.2	3.1 ± 0.2	−26
22:6	2.4	1.6	−33	7.3 ± 0.7	6.6 ± 0.3	−9

[a]Control serum A: cholesterol, 240 mg/dl; triglycerides, 0.90 mg/dl. Control serum B: cholesterol, 240 mg/dl; triglycerides, 1.10 mg/dl. Total FA analyses were performed in triplicate with serum sample and on three different flasks for each fibroblast total FA analysis, and three other different flasks for each fibroblast phospholipid FA analysis (not reported here).

of the level of this FA in the serum used in the culture medium. These observations were most marked in PL.

DISCUSSION

The present investigations were designed to study the role of nutritional and environmental factors on lipid metabolism in two cell types: cultured fibroblasts and fresh leukocytes. Although all cells can synthetize FA de novo, this synthesis is inhibited when FA are present in the culture medium (13); thus the cells preferentially use preformed FA from the medium (14,15). In addition, FA generally incorporated in cells are not altered (16), and elongation and desaturation rates are rather slow compared to the esterification rate (17). This was extensively reviewed by Spector et al. (18).

The analysis of freshly isolated cells such as leukocytes compared with fibroblasts shows that the culture medium greatly influences the FA composition of cells. Many studies have been performed on leukemic cells (9,19,20) and on FA of control leukocytes or lymphocytes, but generally under immune stimulation (21-25). However, our results are in good agreement with their control cellular PL FA analysis.

As a matter of fact, leukocytes freshly isolated from human blood and fibroblasts cultured in medium enriched in HS present a relatively similar FA distribution, even when the drastic difference in their lipid/protein ratios is unexplained. The former contain only slightly more arachidonic acid in their PL. It is obvious that this particular FA composition of cells bathing in similar media but isolated from different tissues can be related to in vivo cellular functions; arachidonic acid can be related to prostaglandin precursors, present in large amounts in immune cells, as this was also observed in endothelial cells richer in arachidonic acid (26).

However, more important differences are obtained in normal fibroblasts cultured in medium enriched in HS or FBS. The same observation was reported with endothelial cells (27), which differed widely in FA composition when cultured in FBS or freshly isolated from the human umbilical cord. It is obvious that the quantity and quality of FA are very different in FBS and HS, i.e., compared to HS, FBS has half as much protein and five times less FA, and it contains less linoleic acid and n-6 metabolites, but is enriched in the n-3 family, SFA and MUFA.

However, even with a different medium, intracellular regulation results in a similar distribution of SFA and

MUFA in the cells and essentially in PL. This is also verified for PUFA, which present some qualitative differences, but a great stability of the n-3 family. This cellular regulation allows a modulation of the incorporation of FA present in the medium, in that cells always present a higher level of SFA than in the medium (FBS or HS), even if the medium contains different concentrations. Cells also always incorporate less linoleic acid than the quantity present in the medium, but inversely they in some way "concentrate" n-6 metabolites. However, the cells essentially reproduce the quantitative and qualitative variations of the medium.

First of all, the modifications induced by different media are detectable on cellular growth, i.e., growth is different following enrichment of the medium with 10% HS or FBS. This should be related first to the HS enrichment in PUFA, which influences the fluidity of cellular membranes and the ability of culture to develop. However, in our study, the difference seems to be due more to the quantity of FA supply than the FA quality of each culture medium. This was demonstrated by comparing cells cultured with iso FA quantity supply (given by HS or FBS) and by analyzing the changes of cultured cells after different periods of growth. Differences observed in the level of FA per unit of cellular protein also seem to be due to the growth state of the cells, since less FA were found in rapidly growing cells, and much less SFA and MUFA. The decrease is detected in neutral lipids, essentially triglycerides. The relative stability of phospholipids in quality and quantity of SFA and MUFA, when the medium is largely modified, has also been observed by other authors, the modification always being more marked in neutral lipids (16,28–31). From the correlative enrichment of cells in FA triglycerides with lower growth (at the beginning of the culture or with medium poorly enriched in serum), it can be hypothesized that this is linked to a rapid uptake of lipid from the medium and that these cells should accumulate in TG pool. This can be secondarily reconverted in another lipid class (in PL) as shown by others (32). The extent of this conversion depends on the cell requirement for growth or different metabolism. TG- or CE-rich lipid droplets were observed in cells cultured with hyperlipemic sera-enriched medium (33–35) or with an excess of FFA or very low density lipoproteins (VLDL) (16,36). As for us, we never found this form of lipid accumulation in our cells cultured with normolipemic sera, even when the medium was 20% enriched. Although quantitative lipid modifications of the medium influence the cellular growth, the growth state of the culture does not influence the capacity of cells to be modified in their FA composition by the medium, since confluent fibroblasts and rapidly growing cells can be modified in the same way (Table 6) when the medium is changed. This suggests a rapid but incomplete FA turnover as shown by other authors on leukemic cells (37,38), fibroblasts (39) and endothelial cells (7).

The major modifications in FA composition induced by different culture media occur in PUFA of PL and essentially in the n-6 family. The n-3 family seems to be less modified by the quality of the medium, and the cells maintain a stable level of docosahexaenoic acid (22:6n-3). A higher ratio of the n-3 family in the medium induces a higher level of eicosa- or docosapentaenoic acid rather than docosahexaenoic acid itself, and vice versa. This was clearly shown by our inversion of the medium on confluent culture cells (Table 6) and by others in the case of essential FA deficiency (40) or FA metabolism analysis of astroblasts (41). This can be related to a blockade of Δ4-desaturase.

Concerning the n-6 family, linoleic acid must be isolated, because its intracellular level depends on the level in the medium, but intracellular n-6 metabolite levels depend on both the level in the medium and the growth state of the cells. Cells show higher levels of n-6 metabolites when grown in media rich in n-6 metabolites and also with a longer bathing in these media. We observed at the same state of growth a relative stability of arachidonic acid levels, regardless of the level in the medium (HS or FBS). But FA resulting from elongation and desaturation of arachidonic acid, docosatetraenoic (22:4n-6) and docosapentaenoic (22:5n-6) acids show levels directly related to their levels in the medium and to the growth state of the culture. The relative stability of cellular arachidonic acid level correlated with the higher level of elongation product (22:4n-6) when the medium was enriched in arachidonic acid was also reported under different culture conditions (16). Since human fibroblasts contain elongation enzymes, it was postulated that some FA taken up from the medium can be elongated when higher levels are available (42). We also observed that the blockade of Δ6-desaturase (lower level of cellular γ-linolenic acid) related to a higher level of linoleic acid induces an elongation process conducing to an enrichment of 20:2n-6 in cells bathing in HS medium. Similar results were obtained with endothelial cells (43) or fibroblasts (16) supplemented with individual FA. These cells apparently express a very low Δ6-desaturase activity when grown in a lipid-rich medium (5,6).

Finally, the quality of FA in the medium influences the cellular PUFA content but, with a low FA quantitative supply, the quality of FA in the medium has a lesser influence on cellular PUFA quality and apparently no influence on SFA content of PL, the major part being located in cellular membranes, which remain very stable under these conditions. Modification of the quantity of FA supply of the medium and also of the cell qualities (strain and growing state) are more important for the distribution of SFA and MUFA in the neutral lipids of cells.

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Short-term Biological Reproducibility of Serum Fatty Acid Composition in Children

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To assess the biological day-to-day reproducibility of the fatty acid composition in serum lipid fractions in children on their habitual diets, fasting serum specimens were collected from healthy 8- to 9-year-old boys at 2-day (n = 21) or 7-day (n = 19) intervals. Percentage compositions of fatty acids in serum cholesteryl esters (CE), triglycerides (TG), free fatty acids (FFA) and phospholipids (PL) were analyzed by gas chromatography. The reproducibility was estimated by calculating linear correlation coefficients between the values obtained at the two time-points. Generally the correlations were higher in CE and PL than in TG and FFA. In CE the 7-day correlations of major fatty acids were only slightly lower than the 2-day ones. In PL the impairment of correlations was somewhat greater than in CE, but in TG and FFA there was a marked drop in reproducibility from 2- to 7-day intervals. The 2-day correlation coefficients of the percentages of linoleate, dihomo-γ-linolenate, arachidonate and eicosapentaenoate in CE were 0.95, 0.84, 0.91 and 0.92, respectively. In PL, the respective values were 0.86, 0.82, 0.95 and 0.90. The present results indicate high short-term biological reproducibility of fatty acid composition in serum CE and PL and clearly lower in those of TG and FFA.
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Dietary survey methods applicable to large population studies generally do not produce data accurate at the individual level, because of inaccuracy of these methods and a large day-to-day variation in dietary intake of most free-living individuals (1,2). To overcome these problems, analyses of the fatty acid (FA) composition of adipose tissue (3,4), serum or plasma lipids (5), red cell membrane (6) and even cheek cell phospholipids (PL) (7) have been used for estimation of the average FA composition of the diet. The composition of FA in serum lipid fractions is altered when the FA composition of dietary fats is changed in intervention studies (8–10), and it has also been found to correlate with the composition of dietary fat in cross-sectional population surveys (11,12). A change in the ratio of polyunsaturated to saturated FA (P/S ratio) of dietary fats induces rapid alterations in the composition of FA in triglycerides (TG) and free fatty acids (FFA), whereas changes in cholesteryl esters (CE) and PL occur more slowly (13–17).

When using single determinations of FA composition as estimates of the composition of dietary fats in free-living subjects or as a measure of adherence to given instructions in dietary interventions (18,19), it is of interest to know the biological day-to-day variation in the FA composition of different serum lipid fractions. In the present work, the FA compositions in serum CE, TG, FFA and PL were analyzed at 2- or 7-day intervals in children on their habitual diets.

SUBJECTS AND METHODS

Venous blood samples were drawn from antecubital vein into Vacutainer tubes from healthy 8- to 9-year-old schoolboys after an overnight fast (12 hr). The sampling was repeated in 21 subjects after a 2-day interval and in 19 subjects after a 7-day interval. After clotting at room temperature, serum was separated, and the aliquots were stored at −70 C until analyzed.

The serum specimens were collected in Turku, Finland, as an extension of the Finnish Multicenter Study on Atherosclerosis Precursors in Children (see ref. 20). The study protocol was approved by the local school board, and an informed consent was obtained from the parents. No dietary advice was given.

Analysis of FA. Lipids were extracted from 0.5-ml aliquots of serum with chloroform/methanol and purified (21). The extract was fractionated on 0.25-mm Silica Gel G thin layer chromatography (TLC) plates using n-hexane/ethyl ether/acetic acid (85:15:1, v/v/v) as developing solvent. The fraction corresponding to PL was located in UV-light, scraped off and transesterified immediately. After removal of PL, the TLC plates were stained with Rhodamin 6-G, and the fractions corresponding to FFA, TG and CE were scraped off. CE were saponified with KOH/ethanol at 85 C for 1 hr, and the FA in all four lipid fractions were esterified with H_2SO_4/methanol at 85 C for 2 hr.

The methyl esters of FA were analyzed in an HP-5880 gas chromatograph using an OV-351 fused silica open tubular capillary column (Nordion Instruments, Helsinki, Finland) and two-step temperature programming from 100 to 240 C. The temperature in the injector and detector was 270 C. A splitless injection technique was used. The results were expressed as percentages of the total area of identified FA from 14:0 to 22:6. Methodological reproducibility was measured by extracting duplicate aliquots of serum and running them through the entire analysis in different batches. The coefficients of variation for individual FA in CE and PL ranged between 0.8% and 10% for most FA (Table 1). In TG and FFA, the coefficients of variation were poorer, and varied between 3% and 20% for most of the fatty acids (Table 2).

RESULTS

The mean percentage compositions of FA in all serum lipid fractions in the total study population of 40 subjects at day 0 are shown in Tables 1 and 2. The mean compositions remained unchanged during the 2- and 7-day intervals (data not shown). The biological reproducibilities of FA percentages were estimated as correlation coefficients between the values measured at the two time-points.

Correlations between the time-points were highest for polyunsaturated and most saturated FA in serum CE and PL (Table 3). In CE the correlations of the major FA decreased only slightly from 2- through 7-day intervals.

TABLE 1

Percentage Compositions and Methodological Reproducibilities of Fatty Acids in Serum Cholesterol Esters and Phospholipids

Fatty acid	Cholesteryl esters			Phospholipids		
	Mean	S.D.	C.V.[a]	Mean	S.D.	C.V.[a]
14:0	0.82	0.24	16.0	0.47	0.13	22.5
16:0	10.55	0.87	3.5	26.94	1.50	3.1
16:1	3.34	1.05	4.3	0.74	0.20	10.7
18:0	1.08	0.23	4.6	15.47	0.94	1.7
18:1	21.47	2.72	1.6	13.20	1.54	1.4
18:2ω6	52.84	5.13	0.8	22.37	3.16	1.3
18:3ω6	0.83	0.41	3.8	n.d[b]		
18:3ω3	0.80	0.22	2.8	0.34	0.10	6.5
20:3ω6	0.66	0.11	3.4	3.23	0.50	2.9
20:4ω6	5.41	0.93	5.1	8.99	1.29	3.4
20:5ω3	1.05	0.33	7.1	1.20	0.34	4.5
22:5ω3	n.d.			1.19	0.19	6.2
22:6ω3	0.64	0.17	9.3	4.71	1.05	7.3
n =	40		20	40		20

[a]Methodological reproducibility of fatty acid analyses measured as coefficient of variation (C.V.) between 20 duplicate samples.
[b]n.d., Not determined.

TABLE 2

Percentage Compositions and Methodological Reproducibilities of Fatty Acids in Serum Triglycerides and Free Fatty Acids

Fatty acid	Triglycerides			Free fatty acids		
	Mean	S.D.	C.V.[a]	Mean	S.D.	C.V.[a]
14:0	1.56	0.96	30.8	1.89	0.79	30.4
16:0	24.48	3.23	4.6	22.89	2.42	8.8
16:1	4.25	1.23	9.0	3.96	1.21	10.0
18:0	4.91	1.08	6.8	13.49	3.88	7.7
18:1	45.29	2.69	2.7	41.13	4.04	3.8
18:2ω6	13.19	4.61	3.8	11.40	2.80	6.0
18:3ω3	1.01	0.32	12.6	1.17	0.36	19.0
20:4ω6	1.30	0.47	10.2	1.22	0.41	12.0
22:6ω3	1.06	0.44	21.2	0.94	0.34	27.6
n =	40		20	40		20

[a]Methodological reproducibility of fatty acid analyses measured as coefficient of variation (C.V.) between 20 duplicate samples.

TABLE 3

Reproducibilities of the Percentage Composition of Fatty Acids in Serum Cholesteryl Esters and Phospholipids at 2- and 7-Day Intervals

Fatty acid	Cholesteryl esters		Phospholipids	
	2 Days	7 Days	2 Days	7 Days
14:0	0.71***	0.44	0.34	0.65**
16:0	0.69***	0.67**	0.84***	0.50*
16:1	0.89***	0.71***	0.57**	0.19
18:0	0.81***	0.72***	0.73***	0.76***
18:1	0.91***	0.82***	0.55**	0.78***
18:2ω6	0.95***	0.80***	0.86***	0.61**
18:3ω6	0.85***	0.59**	n.d.	
18:3ω3	0.91***	0.87***	0.81***	0.60**
20:3ω6	0.84***	0.85***	0.82***	0.67**
20:4ω6	0.91***	0.90***	0.95***	0.83***
20:5ω3	0.92***	0.59**	0.90***	0.55*
22:5ω3	n.d.		0.85***	0.65**
22:6ω3	0.92***	0.79***	0.93***	0.80***
n =	21	19	21	19

Statistical significance of the correlation coefficient: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. n.d., Not determined.

TABLE 4

Reproducibilities of the Percentage Composition of Fatty Acids in Serum Triglycerides and Free Fatty Acids at 2- and 7-Day Intervals

Fatty acid	Triglycerides		Free fatty acids	
	2 Days	7 Days	2 Days	7 Days
14:0	0.71***	0.26	0.16	0.42
16:0	0.68***	0.52*	0.51*	0.76***
16:1	0.57**	0.34	0.61**	0.31
18:0	0.25	0.50	0.53*	0.35
18:1	0.41	0.73***	0.60**	0.43
18:2ω6	0.87***	0.48	0.91***	0.74***
18:3ω3	0.75***	0.47*	0.36	0.64**
20:4ω6	0.82***	0.59**	0.28	0.09
22:6ω3	0.88***	0.71***	0.41	0.36
n =	21	19	21	19

Statistical significance of the correlation coefficient: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

The decrease was somewhat greater in PL, although most of the r-values remained highly significant even at the 7-day interval. The correlation coefficients for linoleic (18:2) and arachidonic (20:4) acids in CE were 0.95 and 0.91 at the 2-day and 0.80 and 0.90 at the 7-day interval, respectively. The respective values for 18:2 and 20:4 in PL were 0.86 and 0.95 at the 2-day and 0.61 and 0.83 at the 7-day interval. There was a trend toward lower correlations in saturated and monounsaturated fatty acids in comparison to polyunsaturated fatty acids; the lowest reproducibilities were for 14:0, 16:0 and 16:1.

The 2-day correlations of polyunsaturated FA in TG were only slightly lower than those in CE or PL, but declined more with time from the 2- to the 7-day period (Table 4). The reproducibilities of saturated and monounsaturated FA in TG were generally low and, especially at the 7-day interval, mostly statistically nonsignificant. With the exception of the 18:2 percentage, most of the correlations of the fatty acids in the FFA fraction were poor already at the 2-day interval and showed no consistent correlations from 2 through 7 days (Table 4).

DISCUSSION

Although the composition of FA is different in each of the four lipid fractions in serum, during constant dietary intake of fats the proportion of a given FA in one fraction is in certain equilibrium with its proportion in other

fractions and in other tissues as well. This is demonstrated by high correlations of the percentages of FA in serum CE with the same FA in other lipid fractions in serum (11,22) or platelet lipids (23). In free-living subjects, however, the diet is never constant. For instance, the P/S ratio of the diet may range from 0.05 to 1.30 between days in an individual's diet during a one-week period (24).

The present results show that the day-to-day variation of FA composition is fairly small in serum CE and PL, but substantially higher in TG and FFA. According to dietary intervention studies, a change in dietary P/S ratio can be demonstrated in serum TG and FFA fatty acids within a few days, whereas the alterations in CE and PL fatty acids occur slowly over a period of one to two weeks (13–17). Thus a substantial part of the observed differences in reproducibility between the lipid classes may be attributable to the insensitivity of CE and PL FA composition to the daily fluctuation in dietary fat intake.

The methodological reproducibility of FA analyses in TG and FFA was poorer than in CE and PL and corresponds to results of other authors (25). Even though the coefficients of variation for the major FA in TG and FFA were satisfactory, the reproducibilities of these FA were of the same general magnitude as the other fatty acids in these fractions, and it seems unlikely that the present low correlations in TG and FFA were due to methodological errors. The concentrations as well as the FA compositions in serum TG and FFA are known to be affected by fasting- vs nonfasting-state differences (26,27). In the present study the subjects fasted for 12 hr before each sampling, but it cannot be ruled out that fasting-state differences contributed to the poor reproducibility in TG and FFA.

The short-term reproducibilities of CE FA percentages in the present study fit well with data from earlier studies on their long-term tracking. The corresponding correlations for 18:2 and 20:4 in CE measured from 18 individuals at a 5-mo interval were 0.76 and 0.74 (22), and the correlations from 805 children and adolescents measured at a 3-year interval were 0.59 and 0.61 (5), respectively.

The respective short-term reproducibilities of serum total cholesterol and TG concentrations in the present study population (n = 54) were 0.82 and 0.47 (28). Thus the reproducibilities of CE FA were of the same magnitude as those of their parent lipid. The slightly better correlations for FA composition than for total concentration of TG are probably caused by the expression of the composition of FA as percentages, which is insensitive to alterations in absolute concentrations.

The present results show a fairly high short-term reproducibility of FA compositions in serum CE and PL and a markedly lower one in TG and FFA. The better reproducibility in CE and PL suggests that these lipid fractions should be preferred when single FA determinations are used as indicators of the composition of dietary fats in free-living subjects or in dietary interventions.

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Effects of Aging on the Composition and Metabolism of Docosahexaenoate-Containing Lipids of Retina

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The amount of docosahexaenoate (22:6n-3)-containing phospholipid species decreases with aging in the rat retina. Most lipids, but especially choline and serine glycerophospholipids, show a significant fall in 22:6n-3, which is not compensated by increases in other polyenoic fatty acids. The decrease not only affects 22:6 but also various very long chain n-3 hexaenoic fatty acids which, in phosphatidylcholine, have up to 36 carbon atoms, and which are probably synthesized by successive elongations of 22:6n-3. The in vitro incorporation of [2-^{3}H]glycerol into retinal lipids indicates that the de novo biosynthetic pathways are not impaired by aging. The incorporation of [1-^{14}C]docosahexaenoate is significantly stimulated into all lipids of aged retinas, but to the largest extent in those showing the largest decreases in 22:6, especially in choline glycerophospholipids. The results indicate that the decreased levels of 22:6 with aging are due not to an impaired activity of the enzymes involved in the synthesis and turnover of phospholipids but to a decreased availability of this polyene in the retina. It is suggested that this may stem from a defect in some of the enzymatic steps that lead to the synthesis of 22:6n-3, probably that catalyzed by Δ_4 desaturase, the effect on longer hexaenes being secondary to the decreased synthesis of 22:6.
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Alterations of the ratios between phospholipid headgroup classes as well as in the quality and proportions of their fatty acyl moieties are known to bring about dramatic changes in the physicochemical properties of biological membranes, along with many of their functions (1). Qualitative or quantitative changes of this type, affecting lipids, may play an important role among the biochemical causes of the insidious deterioration that many cell functions undergo during the process of aging, since lipids make the matrix where a variety of enzymes, receptors and transport systems work. Polyunsaturated fatty acid–containing lipids are especially likely to be involved, since the synthesis of their polar and nonpolar moieties—particularly that of long chain polyenoic fatty acids—needs the concurrence of, and delicate equilibrium between, many enzymatic activities. The retina is an excellent model to study these problems in mammals because its lipids are characterized by a high proportion of such polyunsaturates. The most abundant fatty acids in visual cells of vertebrates are polyenes of the n-3 series, the major representative being 4,7,10,13,16,19-docosahexaenoate (22:6n-3). The retina also contains a whole variety of tetra-, penta- and hexaenoic fatty acids along with 22:6. These include familiar polyenes like 20:4n-6 or 22:5n-3 and various polyenes whose chain lengths range from 24 to 36 carbon atoms (2,3). The latter are highly concentrated in dipolyunsaturated molecular species of phospholipids of photoreceptor membranes, specifically in phosphatidylcholine (PC) (2). This paper is concerned with the effects of aging on compositional and metabolic aspects of retina phospholipids. It is shown that the levels of docosahexaenoate as well as of other n-3 hexaenes are decreased in aged retina glycerophospholipids, especially in those of choline and serine. The in vitro labeling of retina lipids with [2-^{3}H]glycerol and [1-^{14}C]docosahexaenoate is also compared in young and aged animals. The incorporation of this polyenoic fatty acid is shown to be markedly stimulated with aging in most retina lipid classes, but especially in those showing the largest decreases in 22:6 levels.

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MATERIALS AND METHODS

Wistar rats kept under constant environmental conditions and fed for various generations the same pelleted standard chow diet were used for the present experiments. No sex-related differences were found in the effect of aging on lipids. Rats aged 2–3 and 26–27 mo were killed by decapitation, their eyes were rapidly enucleated, and their retinas were dissected under a magnifying glass. Some of the retinas were immediately homogenized with chloroform/methanol and were destined to lipid and fatty acid composition analysis. Others were incubated with [2-^{3}H]glycerol and 4,7,10,13,16,19-[1-^{14}C]docosahexaenoic acid ([^{14}C]22:6) to determine the labeling of their lipids with these precursors. For this purpose, the retinas were preincubated for 5 min at 37 C in 0.8 ml of a bicarbonate-based ionic medium, pH 7.4, containing 2 mg/ml of glucose (4), after which 0.2 ml of the same medium containing the precursors (5 nmol[^{14}C]22:6 and 0.16 nmol [^{3}H]glycerol) was added, and incubations (37 C) proceeded for the periods indicated in Results. At the end of incubations, 9 ml of ice-cold medium was added and the tubes containing the retinas were centrifuged (10 min at 10,000 rpm). After supernatants were discarded, the pellets were extracted with chloroform/methanol. Protein was determined according to the method of Lowry et al. (5).

The extracts from labeled and unlabeled samples were partitioned and washed according to Folch et al. (6), and the lipids were resolved by means of thin layer chromatography (TLC) (7). After development, the lipid spots were located by exposing the plates to I_2 vapors and subjected to phosphorus analysis (7). The spots containing [^{3}H]- and [^{14}C]-labeled lipids were scraped from TLC plates and transferred to vials containing 0.4 ml water. Ten ml of a solution containing 0.4% Omnifluor (New England Nuclear, Boston, Massachusetts) and 20% Triton X-100 in toluene was then added, and radioactivity was measured by liquid scintillation counting. The [2-^{3}H]glycerol used (sp act 10 Ci/mmol) was from New England Nuclear, and the [1-^{14}C]docosahexaenoate (sp act 40 μCi/μmol) was provided by H. Sprecher, Ohio State University (Columbus, Ohio).

For fatty acid analyses, the lipids were isolated as before, the spots were located under UV light after spraying with dichlorofluorescein, and methyl esters were prepared (under N_2) by BF_3-catalyzed methanolysis (8). The latter were analyzed by gas liquid chromatography (GLC). Two glass columns (2 m × 2 mm id) packed with 15% OV-275 on 80–120 Chromosorb WAW (Varian, Sunnyvale, California) were connected to two flame ionization detectors operated in the dual-differential mode. A linear (5 C/min) temperature program, starting at 160 C and ending at 220 C, was used, and the injector and detector temperatures were 220 C and 230 C, respectively. To obtain the data in Table 3, after reaching 220 C the oven temperature was held constant at that value for 45 min to permit the analysis of very long chain polyenoic fatty acids (2).

RESULTS

Effects of aging on retina phospholipids. The concentration of phospholipids, expressed as the amount of lipid phosphorus per mg of retina protein, was lower by a factor of about 20% in retinas from aged rats than in those from young animals (Table 1). Such a difference in total lipid content was not significant statistically, as when the levels of individual phospholipids were considered. The average amounts of protein were 0.70 ± 0.11 and 0.64 ± 0.11 mg per retina in samples from young and aged retinas, respectively, while those of total phosphorus in lipid extracts from the same samples were 250 ± 34 and 189 ± 25 nmol, respectively. While the average decrease of 8% in protein was not statistically significant, that of 24% in lipid phosphorus was $P < 0.05$. Hence, the contents expressed as lipid/protein ratios in Table 1 show no large differences in lipid concentration because both protein and lipid are affected by aging, but it is apparent from the above results that lipids tend to be decreased relatively more than proteins. When the phospholipid composition (%) was analyzed, again no significant differences due to aging were readily apparent (Table 1). However, some interesting trends were observed by calculating the ratios between individual phospholipids. Most of the age-related differences in total phospholipid concentration were contributed by decreases in the three major phospholipids: phosphatidylcholine, ethanolamine glycerophospholipids (EGP) and phosphatidylserine (PS), which resulted in changes of certain phospholipid ratios. Among choline phospholipids, for instance, the amount of PC was decreased to a larger extent than that of sphingomyelin (Sph, unchanged), giving rise to a significantly lower PC/Sph ratio in aged retinas. Among acidic phospholipids, PS was more decreased than phosphatidylinositol (PI), which also resulted in a significant difference in the PS/PI ratio with age. The PC/PS ratio was similar in control and aged retinas, indicating that both were diminished to the same extent, and the PC/EGP ratio was increased. The fatty acid composition of retina glycerophospholipids was next examined in search for the possible involvement of polyenoic fatty acids in these effects, since it was apparent that the largest decreases affected those lipids that contribute the largest amounts of the most highly unsaturated fatty acids to retinal membranes.

Fatty acids of retina glycerophospholipids. The most conspicuous effect of aging on the fatty acid composition of lipids was a general tendency to a decreased proportion of 22:6n-3 (Table 2). Its percentage was about 30%, 10% and 13% lower in PC, EGP and PS, respectively, in aged than in young animals. If one considers that the amounts of these phospholipids were also lower (Table 1), it may be estimated that the amount of 22:6 per mg of protein decreased by about 42%, 31% and 30% as a consequence of the changes in such lipids (Table 4). It is evident from Tables 2–4 that no fatty acid was synthesized in retina to compensate for this decrease in 22:6, the slight increases observed in the percentages of some fatty acids being only relative to this diminution. The average unsaturation of lipids tended to decrease (Table 2), the most significant effect ($P < 0.05$) being observed for PC and PS.

The fatty acid composition of PC is expanded in Table 3

TABLE 1

Content and Composition of Phospholipids in Rat Retina

	Controls		Aged		Controls/aged
PC		159.1 ± 21.8 (45.7 ± 0.9)		131.7 ± 20.2 (45.3 ± 0.6)	0.83
EGP	315 ± 47	115.1 ± 15.2 (33.1 ± 0.7)	254 ± 39	89.1 ± 14.3 (31.6 ± 1.4)	0.77
PS		40.8 ± 6.1 (11.6 ± 0.5)		32.8 ± 3.9 (11.3 ± 0.4)	0.80
PI		16.7 ± 3.3 (4.8 ± 0.2)		16.2 ± 0.9 (5.7 ± 0.5)	0.97
PA	32 ± 7	5.6 ± 1.5 (1.6 ± 0.2)	34 ± 6	5.2 ± 1.6 (1.6 ± 0.4)	0.91
Sph		5.7 ± 1.9 (1.6 ± 0.2)		7.8 ± 0.6 (2.6 ± 0.4)	1.37
DPG		4.6 ± 3.0 (1.3 ± 0.5)		5.3 ± 4.0 (1.7 ± 0.7)	1.15
Total phospholipid		350.0 ± 52.0		289.0 ± 47.0	
PC/EGP		1.38 ± 0.04		1.48 ± 0.02*	
PS/PI		2.46 ± 0.20		2.01 ± 0.20*	
PC/Sph		29.40 ± 6.50		16.95 ± 2.24*	

PC, phosphatidylcholine; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; Sph, sphingomyelin; DPG, diphosphatidylglycerol. The figures depict the amount of phospholipid as nmol/mg protein. The percentage composition is given in parentheses. Both are presented as mean values ± S.D. from 4 samples (each containing 6–8 retinas) from control (2.5 mo) and aged (26.5 mo) rats. Lipids were separated by two-dimensional thin layer chromatography. The ratios between lipids were calculated for individual samples and then averaged. *, Significant differences with respect to controls ($P < 0.05$ or lower).

TABLE 2

Fatty Acid Composition (Mol%) of Rat Retina Glycerophospholipids

	Phosphatidylcholine		Ethanolamine glycerophospholipids		Phosphatidylserine		Phosphatidylinositol		Phosphatidic acid	
	2.5 mo	26.5 mo	2.5 mo	26.5 mo	2.5 mo	26.5 mo	2.5 mo	26.5 mo	2.5 mo	26.5 mo
14:0	0.3 ± 0.1	0.4 ± 0.1	0.7 ± 0.8	0.6 ± 0.6	0.1 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	2.0 ± 1.9	0.7	1.0
15:0	0.3 ± 0.0	0.6 ± 0.1	0.9 ± 1.0	0.4 ± 0.4	0.1 ± 0.1	0.2 ± 0.2	—	0.2 ± 0.2	0.5	0.6
16:0	34.3 ± 0.7	35.7 ± 1.0	6.8 ± 1.0	8.6 ± 0.5	0.9 ± 0.3	1.9 ± 1.4	8.9 ± 1.5	10.7 ± 3.3	13.4	19.2
17:0	1.9 ± 0.2	1.6 ± 0.1	—	—	0.2 ± 0.1	0.9 ± 0.9	—	1.1 ± 0.6	2.6	2.9
18:0	18.5 ± 2.3	18.3 ± 1.6	30.3 ± 2.8	32.1 ± 1.3	37.2 ± 2.9	40.8 ± 2.4	35.3 ± 0.6	36.0 ± 1.4	33.3	32.4
18:1	18.3 ± 0.4	22.2 ± 1.5*	4.8 ± 0.4	5.4 ± 0.5	2.1 ± 0.3	3.8 ± 1.0*	4.3 ± 0.3	4.8 ± 1.4	11.4	11.7
18:2n-6	0.9 ± 0.1	1.1 ± 0.6	0.4 ± 0.1	0.4 ± 0.3	0.1 ± 0.04	0.2 ± 0.1	0.2 ± 0.5	0.2 ± 0.2	0.7	0.5
20:1	0.4 ± 0.1	0.3 ± 0.01	0.1 ± 0.1	0.1 ± 0.03	0.1 ± 0.02	0.2 ± 0.2	0.1 ± 0.05	0.1 ± 0.1	0.2	0.2
20:3n-6	0.1 ± 0.05	0.1 ± 0.05	0.2 ± 0.03	0.1 ± 0.04	0.1 ± 0.03	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.3	0.3
20:4n-6	5.4 ± 0.6	5.7 ± 0.5	10.7 ± 0.9	12.0 ± 1.6	3.8 ± 0.2	4.6 ± 0.3	46.7 ± 1.7	40.6 ± 2.4*	10.8	18.5
20:5n-3	0.02 ± 0.01	0.1 ± 0.01	0.1 ± 0.05	0.2 ± 0.04	0.1 ± 0.1	0.1 ± 0.02	0.5 ± 0.3	0.8 ± 0.2	0.4	0.3
22:4n-6	0.4 ± 0.04	0.5 ± 0.1	2.3 ± 0.2	2.0 ± 0.1	3.4 ± 0.4	2.6 ± 0.1	0.3 ± 0.1	0.3 ± 0.2	1.7	0.8
22:5n-6	0.4 ± 0.1	0.1 ± 0.04*	1.2 ± 0.2	0.4 ± 0.1*	2.0 ± 0.2	0.6 ± 0.2*	0.3 ± 0.2	—*	0.5	0.5
22:5n-3	0.2 ± 0.03	0.3 ± 0.01	0.5 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	1.1 ± 0.2		0.1 ± 0.1	0.7	0.2
22:6n-3	17.0 ± 1.5	11.9 ± 0.9*	40.3 ± 1.7	36.0 ± 2.0*	45.3 ± 1.8	39.3 ± 2.4*	3.1 ± 1.3	2.9 ± 0.2	22.2	10.3
24:5n-3	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.2	0.7 ± 0.2	1.6 ± 0.2	1.6 ± 0.2	—	—	0.6	0.4
24:6n-3	0.3 ± 0.1	0.1 ± 0.02*	0.5 ± 0.2	0.4 ± 0.4	2.2 ± 0.3	1.6 ± 0.2*	—	—	0.6	0.3
C_{26}-C_{36} PUFA	1.2	0.7	—	—	—	—	—	—	—	—
Average unsaturation	1.6 ± 0.1	1.3 ± 0.05*	3.0 ± 0.2	2.9 ± 0.2	3.4 ± 0.1	3.0 ± 0.1*	2.2 ± 0.1	1.9 ± 0.1	2.1	1.6

Lipids were preparatively isolated by means of thin layer chromatography and their fatty acid composition was analyzed by gas liquid chromatography of fatty acid methyl esters. Results are presented as mean values ± S.D. from 3 samples, each containing 6–8 retinas. Average unsaturation represents the average number of double bonds in fatty acids per mole of lipid. *, Signiaficant effects of aging ($P < 0.05$ or less). Phosphatidic acid was analyzed after combining the three samples of each group.

TABLE 3

Tetra-, Penta- and Hexaenoic Fatty Acids of Rat Retina Phosphatidylcholine

Fatty acid	Controls (mol% × 10)	Aged (mol% × 10)
Up to 20:4n-6	750.75	804.40
20:4n-6	54.00	56.70
20:5n-3	0.20	1.40
22:4n-6	4.10	4.90
22:5n-6	3.50	1.30
22:5n-3	1.80	2.50
22:6n-3[a]	170.10	118.90
24:5n-3	1.20	1.10
24:6n-3[b]	2.70	1.40
26:5n-3	0.06	0.15
26:6n-3[c]	0.40	0.30
28:5n-3	0.06	0.06
28:6n-3	0.21	0.15
30:4n-6	0.02	0.01
30:5n-3	0.06	0.06
30:6n-3	0.06	0.02
32:4n-6	0.08	0.10
32:5n-3	1.10	0.80
32:6n-3	5.00	3.20
34:4n-6	0.20	0.20
34:5n-3	0.70	0.60
34:6n-3	2.90	1.70
36:4n-6	0.01	0.01
36:5 + 36:6n-3	0.08	0.06
Total VLCPUFA		
Mol%	1.56	0.99
Wt%	2.41	1.53

VLCPUFA, very long chain polyunsaturated fatty acid. Methyl esters from the phosphatidylcholine samples whose composition is shown in Table 2 were combined and injected in a concentrated form to facilitate detection and quantitation of VLCPUFA. Total VLCPUFA is the sum of C_{24} to C_{36} polyenes.

[a,b,c]Coelute with minor amounts of 24:4n-6, 26:4n-6 and 28:4n-6, respectively.

to show the series of very long chain polyenoic fatty acids that elute after 22:6 (2). C_{24}–C_{36} polyenoic fatty acids made up 2.4% of the fatty acid weight of PC from rat retina. This may seem a minor proportion, but such very long chain polyenes are specifically esterified to some of the PCs (the dipolyunsaturated molecular species of the phospholipid) present in photoreceptor membranes (which in turn are a small fraction of the total membranes of entire retina) (2). Hence, the proportion of very long chain polyenes in the entire retina PC is small because they are "diluted" with the PCs from many cell membranes that do not contain such fatty acids. Even so, some very long chain polyenes, like 32:6 and 34:6, occur in higher proportions than some of the polyenes listed between 20:4n-6 and 22:6n-3 shown in Table 3.

The sum of very long chain polyenoic fatty acids of PC decreased in aged retinas, but some of them contributed more than others to this effect (Table 3). Thus, consistent with the effect observed for the "shortest" hexaene (22:6n-3), all longer n-3 hexaenes were also decreased. Very long chain (n-3) pentaenes and (n-6) tetraenes were less affected, as was also the case for the shortest polyenes of the respective series, such as 20:5n-3 and 20:4n-6. This fits into the general tendency observed for the polyenoic fatty acids of all retinal glycerophospholipids, which are summarized in Table 4: a) the ratio between n-3 and n-6 polyenes decreased with age, not due to an increase in n-6, but to a decrease in n-3 polyenes; b) among n-3 polyenes, the n-3 hexaene/pentaene ratio decreased with age, due primarily to a decrease in n-3 hexaenes; c) the absolute amounts of total polyenes as well as their percentages in lipids decreased with age, showing that no polyene was synthesized to substitute for the depleted 22:6; d) the effect on polyenes mainly accounted for the

TABLE 4

Summary of the Effects of Aging on Polyenoic Fatty Acids of Retina Glycerophospholipids

	PC (mol%)		EGP (mol%)		PS (mol%)		PI (mol%)		PA (mol%)	
	2.5 mo	26.5 mo	2.5 mo	26.5 mo	2.5 mo	26.5 mo	2.5 mo	26.5 mo	2.5 mo	26.5 mo
Total polyenes	26.0	20.8*	55.8	52.9	59.6	51.9*	51.2	45.0*	38.5	32.1
n-6 Polyenes	7.3	7.5	14.1	14.9	9.4	8.2*	47.6	41.2*	14.0	20.6
n-3 Polyenes	18.7	13.3*	41.7	38.0	50.2	43.7*	3.6	3.8	24.5	11.5
n-3/n-6 Polyenes	2.6	1.8*	3.0	2.6	5.3	5.3	0.1	0.1	1.8	0.6
n-3 Pentaenes	0.5	0.7	0.9	1.6*	2.7	2.8	0.5	0.9	1.7	0.9
n-3 Hexaenes	18.2	12.6*	40.8	36.4	47.5	40.9*	3.1	2.9	22.8	10.6
n-3 Hexa/pentaenes	36.4	18.0*	45.3	22.8*	17.6	14.6*	6.2	3.2	13.4	11.8
Total polyenes (nmol/mg protein)[a]	20.7	13.7*	32.1	23.6	12.2	8.5*	4.3	3.6	1.1	0.8
22:6n-3 (nmol/mg protein)[a]	13.5	7.8*	23.2	16.0*	9.2	6.4*	0.3	0.2	0.6	0.3

PC, phosphatidylcholine; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid. Total polyenes is the sum (mol%) of all polyenes detected in fatty acid composition analyses. The fatty acids were grouped as indicated to calculate the ratios. *, Significant effects of aging ($P < 0.05$ or less).

[a]Amounts estimated as (nmol fatty acid(s)/nmol lipid) × (nmol lipid/mg protein).

tendency shown by the major phospholipids to decrease their concentrations in aged retinas (Table 1), since other fatty acids were affected to lower extents. The incorporation of [1-^{14}C]docosahexaenoate in retina lipids was then studied to determine whether the age-dependent defect in 22:6 was due to an impairment of the enzymes involved in the turnover of their 22:6-containing species and was compared to that of [2-^{3}H]glycerol in search for possible effects of aging on the de novo synthesis of such lipids.

General features of the incorporation of [^{3}H]glycerol and [^{14}C]docosahexaenoate in retina lipids. After incubating retinas with these precursors, the incorporation of [^{14}C]22:6 was faster and more efficient than that of [^{3}H]glycerol (Table 5). One of the important factors determining this result was that the fatty acid was likely to be present in much larger concentrations in the tissue than glycerol, simply because it is relatively more "soluble" in the retinal lipid membranes than in the medium, as opposed to the water-soluble precursor. In the present experiments, the concentration of free [^{14}C]22:6 in the incubation medium was indeed higher than that of glycerol (5 vs 0.16 nmol, respectively, were offered to the tissue). To account for this difference in the concentration of precursors, the amount of incorporated glycerol was in all cases multiplied by a factor of 31.25, but even after this correction the ^{14}C/^{3}H ratios were always greater than 1 (Tables 5–7). Such correction, of course, cannot level off the possible differences in intracellular concentrations attained (which are ignored) nor the possible differences in compartmentation.

The incorporation of [^{3}H]glycerol into rat retina lipids followed the general routes through which the de novo synthesis of glycerol-containing lipids occurs in most vertebrate tissues. Thus, at early incubation times (10 min), phosphatidate was the lipid concentrating most of the label, followed by diacylglycerols and phosphatidylinositol (PI) (Table 6). While PI rapidly attained a plateau, the synthesis of PC continued throughout the incubation interval, this lipid being the predominant product at long

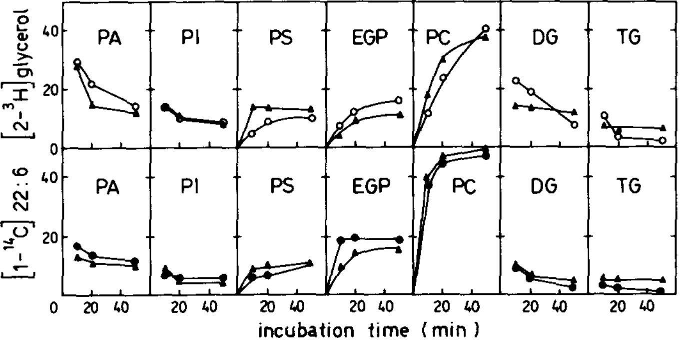

FIG. 1. Comparison of the time-course of the distribution of [^{3}H]-glycerol and [^{14}C]docosahexaenoate among lipids of control (circles) and aged (triangles) retinas. Results are percentages of the total incorporated activity in each case, calculated from the mean values in Tables 5 and 6. PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; EGP, ethanolamine glycerophospholipids; DG, diacylglycerols; TG, triacylglycerols.

incubation times. Most of the difference in total [^{3}H]-glycerol incorporated between 20- and 50-min incubations (Table 5) was accounted for by the labeling of PC (Table 6). The relationships between the various lipids are readily apparent when represented on a relative basis (Fig. 1). The contribution of phosphatidate and diacylglycerols to the total label in lipids decreased with time, in favor of the final products.

A large proportion of the total free [^{14}C]22:6 added to the media was recovered in the (comparatively very small volume of) tissue after incubations (Table 5). The esterification of free 22:6 by the retina was highly efficient, as indicated by the fact that a large proportion of the total label in the tissue was recovered in lipids (Table 5). In contrast to the redistributions observed for the [^{3}H] label among lipids (Fig. 1), [^{14}C]22:6 was predominantly incorporated in PC at all incubation times analyzed (40–50% of the esterified label). This indicates that most of the

TABLE 5

Effect of Aging on the In Vitro Incorporation of [1-^{14}C]Docosahexaenoate and [2-^{3}H]Glycerol in Rat Retinal Lipids

	Incubation time (min)	[1-^{14}C]Docosahexaenoate					
		Free	Esterified	Total [^{14}C]22:6[a]	Esterified/free	[2-^{3}H]Glycerol	[14]C/[3]H[b]
Control rats	10	952 ± 125	45 ± 20		0.05	9 ± 1	5.0
	20	914 ± 135	205 ± 15	1038 ± 70	0.22	78 ± 8	2.6
	50	769 ± 202	229 ± 20		0.29	106 ± 8	2.2
Aged rats	10	972 ± 100	117 ± 11		0.12	20 ± 7	5.9
	20	746 ± 120	352 ± 13	1087 ± 12	0.47	79 ± 17	3.3
	50	707 ± 174	369 ± 26		0.52	110 ± 14	3.3

The incorporations are expressed as pmol [^{14}C]22:6 or [^{3}H]glycerol in lipids/mg of retinal protein (mean values ± S.D. from 3 samples, each containing the two retinas of a rat). Control and aged retinas were 2.5 and 26.5 months old, respectively. The retinas were incubated for the specified intervals with both precursors, added simultaneously. The amount of incorporated [2-^{3}H]glycerol was multiplied by a factor of 31.25 to account for the difference in the amounts of [^{14}C]22:6 and [^{3}H]glycerol added to the incubation media (5 and 0.16 nmol, respectively).

[a]Free + esterified.

[b]Esterified [1-^{14}C]docosahexaenoate/[2-^{3}H]glycerol.

TABLE 6

Labeling of Retina Lipids by [2-^{3}H]Glycerol and [1-^{14}C]Docosahexaenoate

	[2-^{3}H]Glycerol			[1-^{14}C]Docosahexaenoate		
	10 min	20 min	50 min	10 min	20 min	50 min
Control rats						
Phosphatidylcholine	1.1 ± 0.1	19.2 ± 1.6	43.2 ± 3.7	17.3 ± 0.9	92.3 ± 10.9	109.2 ± 1.3
Ethanolamine glycerophospholipids	0.7 ± 0.1	9.4 ± 0.5	17.2 ± 3.7	8.4 ± 1.0	41.2 ± 3.0	43.2 ± 3.2
Phosphatidylserine	0.5 ± 0.5	7.1 ± 3.3	10.2 ± 1.1	2.9 ± 0.9	13.7 ± 5.7	25.5 ± 3.0
Phosphatidylinositol	1.3 ± 0.5	7.8 ± 2.8	9.1 ± 0.4	3.1 ± 1.7	12.5 ± 2.3	14.6 ± 4.8
Phosphatidic acid	2.7 ± 0.2	17.0 ± 2.9	15.3 ± 2.0	7.5 ± 1.0	27.7 ± 8.8	26.8 ± 4.1
Diacylglycerols	2.2 ± 0.6	14.9 ± 0.1	8.5 ± 0.6	4.2 ± 3.1	12.3 ± 0.6	7.1 ± 0.4
Triacylglycerols	1.0 ± 1.0	3.2 ± 1.2	2.8 ± 2.6	1.9 ± 0.6	5.1 ± 2.3	2.7 ± 2.3
PC/EGP ratio	1.5 ± 0.1	2.0 ± 0.5	2.5 ± 0.8	2.1 ± 0.3	2.3 ± 0.3	2.5 ± 0.2
Aged rats						
Phosphatidylcholine	3.6 ± 0.2	24.1 ± 0.7	41.5 ± 0.4	47.7 ± 4.5	164.5 ± 20.4	182.4 ± 4.4
Ethanolamine glycerophospholipids	0.9 ± 0.2	7.1 ± 0.1	12.0 ± 0.8	12.0 ± 4.0	52.8 ± 7.8	52.8 ± 4.3
Phosphatidylserine	2.8 ± 0.1	10.0 ± 2.0	14.5 ± 2.3	10.4 ± 0.9	37.7 ± 7.4	40.2 ± 9.2
Phosphatidylinositol	2.7 ± 0.8	8.3 ± 2.5	8.8 ± 2.1	10.6 ± 3.4	18.7 ± 2.5	14.0 ± 4.4
Phosphatidic acid	5.6 ± 0.4	11.6 ± 2.9	13.4 ± 2.3	15.1 ± 1.0	38.8 ± 6.0	35.0 ± 2.2
Diacylglycerols	2.9 ± 1.2	10.9 ± 0.4	13.0 ± 3.6	12.2 ± 4.8	20.1 ± 6.0	22.8 ± 8.8
Triacylglycerols	1.5 ± 1.1	4.8 ± 4.7	7.1 ± 3.6	6.8 ± 6.9	20.1 ± 21.0	21.7 ± 13.6
PC/EGP ratio	3.9 ± 0.5	3.4 ± 0.03	3.5 ± 0.5	4.0 ± 1.8	3.2 ± 0.8	3.1 ± 0.2

Lipids labeled in the retina with both radioactive precursors were separated by thin layer chromatography. The figures represent the incorporation into each lipid class (pmol/mg retinal protein). Details as in Table 5.

available 22:6 was incorporated directly into preexisting PC molecules through acyl exchange reactions, rather than through the slower neobiosynthetic reactions. However, label from the fatty acid was in all glycerolipids, including phosphatidate (PA) and diacylglycerol, for part of the label in major phospholipids, particularly at long incubation times, may be contributed by (the incorporation of) these labeled intermediates. The specific radioactivity of PA was the highest among phospholipids with either glycerol or 22:6 (Table 7), which supports this possibility.

The differences in rates of incorporation of both precursors become even larger in favor of 22:6 if one takes into account that while [^{3}H]glycerol is introduced in the skeleton of all kinds of lipid molecular species from disaturated to dipolyunsaturated (9), [^{14}C]22:6 only labels

TABLE 7

Aging Effects on Average Specific Radioactivities of Retina Glycerophospholipids

	Incubation time (min)	[2-^{3}H]Glycerol					[1-^{14}C]Docosahexaenoate					[1-^{14}C]Docosahexaenoate				
		PC[a]	EGP	PS	PI	PA	PC	EGP	PS	PI	PA	PC	EGP	PS	PI$\times 10^{-3}$	PA$\times 10^{-3}$
		(fmol ^{3}H or ^{14}C/nmol lipid P)[b]										(fmol [^{14}C]22:6/nmol 22:6)[c]				
Control rats	10	7	6	12	78	474	109	73	171	185	1316	320	90	80	3.0	3.0
	20	121	82	174	470	2980	608	360	335	750	4860	1790	450	370	12.1	11.0
	50	272	149	250	545	2680	720	374	625	870	4700	2120	465	690	14.0	10.6
Aged rats	10	27	10	85	167	1077	362	134	317	650	2904	1520	185	405	11.2	14.1
	20	185	80	305	512	2230	1250	593	1150	1150	7460	5250	825	1465	19.9	36.2
	50	315	135	442	543	2580	1500	660	1225	864	6730	6300	915	1560	14.9	32.7
Aged rats/ control rats	10	3.9	1.7	7.0	2.1	2.3	3.3	1.8	4.5	3.5	2.2	4.8	2.1	5.0	3.7	4.7
	20	1.5	1.0	1.8	1.1	0.7	2.1	1.6	3.4	1.5	1.5	2.9	1.8	2.3	1.6	3.3
	50	1.2	0.9	1.8	1.0	1.0	2.1	1.8	2.0	1.0	1.4	3.0	2.0	2.3	1.1	3.1

[a]PC, phosphatidylcholine; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid.
[b]The specific radioactivity of each lipid class was calculated as (fmol ^{3}H or ^{14}C in lipid/mg protein)/(amount of lipid/mg protein).
[c]Specific radioactivities of 22:6-containing species within each lipid class, calculated as (fmol [^{14}C]22:6/nmol lipid [fmol ^{3}H or ^{14}C in lipid/mg protein])/(nmol of 22:6/nmol lipid).

the 22:6-containing species of each retinal lipid (namely hexaenoic and dipolyunsaturated species [10]). The highest specific radioactivities, whether expressed per mole of lipid or per mole of 22:6 in lipid (Table 7), were attained by PA and PI with both precursors. Among the three major membrane phospholipids (PC, EGP and PS), the highest turnover rates of 22:6 (i.e., the exchange of labeled for unlabeled fatty acid) were attained by docosahexaenoate-containing species of PC. This is noteworthy, since both PS and EGP are richer in 22:6 than PC (Table 2).

Effects of aging on retinal lipid labeling with [^{3}H]-glycerol and [^{14}C]22:6. In the presence of equal amounts of free [^{14}C]22:6, retinas from aged rats incorporated about twice as much fatty acid in their lipids than those from young animals (Table 5). The labeling of all lipids by the fatty acid was stimulated (Tables 6 and 7), but PC and PS showed the largest increases in relation to other lipids (Fig. 1).

The incorporation of [^{3}H]glycerol was much less affected by aging than that of [^{14}C]22:6 (Table 5). No significant differences were observed in the total glycerol incorporated after 20- and 50-min incubation, even though a stimulation was apparent at short incubation times (10 min). This may have been provoked by the presence of unesterified [^{14}C]22:6 in the tissue since, coincidentally, the lipids whose labeling with [^{3}H]glycerol was most stimulated (especially early on) were also PC and PS (Table 6, Fig. 1). Of the two zwitterionic phospholipids, synthesis of PC was relatively more stimulated than that of EGP (see the PC/EGP ratios in Table 6). Similarly, of the two acidic phospholipids, labeling of PS increased more than that of PI (note that the labeled PS/PI ratios were 0.4, 0.9 and 1.1 for controls and 1.0, 1.2 and 1.6 for aged retinas after 10-, 20- and 50-min incubations, respectively, with glycerol). Such changes were not due to a depressed synthesis of EGP or PI (Tables 6 and 7) but to a relatively faster formation of PC and PS.

The specific radioactivities of [^{14}C]22:6-labeled lipids were in all cases larger in the retinas from the aged rat group (Table 7). The selective stimulation of the turnover of 22:6 in PC and PS became even more apparent when the specific radioactivities were expressed as fmol [^{14}C]-22:6 incorporated per mole of 22:6 in each lipid (Table 7), since the latter tended to decrease in all lipids of aged retinas (Table 2) but especially in these two phospholipid classes.

DISCUSSION

A marked decrease in the levels of 22:6n-3 along with other polyenoic fatty acids occurs in the glycerophospholipids of retina as a consequence of aging (Table 4). The phospholipids containing the largest percentages of docosahexaenoate (PC, EGP and PS) are those that exhibit the largest quantitative decreases of this fatty acid (Tables 2–4). This is apparently not compensated by increases in other fatty acids and is manifested in the tendency these phospholipids show to decrease their concentrations when they are expressed as nmol lipid/mg of retinal protein (Table 1). Phospholipids like PI and DPG, which contain much lower levels of 22:6 and other n-3 polyenoic fatty acids, are virtually unchanged, as is also the case with Sph (Table 1), which does not contain any polyenes. It is apparent that aging decreases the ability of retina to make 22:6-containing species of phospholipids. When retinas are incubated with [1-^{14}C]docosahexaenoate, however, it is observed that the esterification of the fatty acid into lipids is markedly stimulated with aging (Table 5). The lipids whose labeling is most stimulated are PC and PS (Tables 6 and 7), precisely the classes undergoing the largest decreases in 22:6n-3, as well as in longer n-3 hexaenoic fatty acids (Tables 2 and 3).

The incorporation of [^{3}H]glycerol and [^{14}C]22:6 into lipids shows that the de novo biosynthetic machinery as well as the enzymatic mechanisms involved in the turnover of 22:6 in preexisting phospholipid molecules are not responsible for the observed decreases in docosahexaenoate. On the contrary, aged retinas seem to work as

though they were more "avid" for 22:6 than their younger counterparts (Tables 5–7), i.e., as though they had developed a higher affinity for the fatty acid. The results allow us to conclude that the levels of 22:6-containing species of lipids are decreased during aging simply because there is less 22:6 available in the retina, since when the fatty acid is provided such enzymes work to attain, and even surpass, their fullest capacity.

That the decrease of 22:6 in lipids is not due to phospholipase A_2 activation during aging is supported by the fact that the levels of free fatty acids did not differ between young and aged retinas. The total free fatty acid pool size in the retinas whose composition is shown in Tables 1 and 2 was 16.2 ± 3.1 and 17.9 ± 3.7 nmol/mg of protein, respectively, of which only 1.8 ± 0.2% and 2.3 ± 1.1% was 22:6n-3, and 4.3 ± 1.4% and 1.7 ± 0.4% was 20:4n-6, respectively (mol% of the total unesterified fatty acids). Even when these are not strictly "endogenous" levels and percentages of free fatty acids, since these are known to be quickly released from excitable tissue lipids due to postdecapitation ischemia (11), no significant effect of age was observed, i.e., no accumulation of polyunsaturated fatty acids occurs in aged retinas, which may account for the decreases observed in phospholipids. Therefore, neither the synthetic nor the degradative processes of phospholipids themselves are responsible for the decreased levels of polyenoic fatty acids displayed in their composition.

There are at least two possibilities to explain the decreased levels of polyenoic fatty acids in lipids of aged retinas: either they are destroyed at higher rates, or they are synthesized less efficiently. Concerning the first alternative, reactions leading to the formation of several peroxidation products and free radicals from polyunsaturated fatty acids may be involved. In fact, these deleterious products do accumulate in senescent tissues, and a debilitation of the defense mechanisms of cells to dispose of them has been proposed to be an important factor contributing to the process of aging (12–14). The susceptibility of lipids to peroxidation runs parallel to their content in polyenoic fatty acids, which makes brain, and especially retina, lipids particularly prone to such damaging reactions. Docosahexaenoate is highly labile, since the larger the number of double bonds, the larger the number of possible hydroperoxides that may be produced, and the greater the rate constants of the reactions leading to free radical formation (15). Products of lipid peroxidation in turn may react with many cell molecules (13,16), giving rise to lipid-lipid, lipid-protein and protein-protein crosslinking. Such crosslinked products may contribute to the decrease in fluidity observed in many cell membranes during aging (17,18). Interestingly, the microviscosity of synaptosomal cortex membranes increases with age, the increase being higher for the hydrophobic than the hydrophilic region of the membrane, which supports this possibility (19). It is obvious at present that many chemical processes of completely differing natures may contribute during aging, with effects that are all translated as a change in this bulk physical property of membranes, including increases in cholesterol content, decreases in unsaturated fatty acids, alterations in phospholipid headgroup ratios, formation of crosslinked products and others.

Concerning the second possibility, i.e., that decreased levels of polyenoic fatty acids in retinal lipids may result from a decreased rate of synthesis of these highly unsaturated acyl moieties, two possibilities may be considered: first, that the enzymes that synthesize them (from essential fatty acids of the n-3 and n-6 series) work normally, but lower amounts of such precursors are available, and second, that such precursors are available, but some of the enzymatic steps involved in their further conversions are impaired. It is well established that the synthesis of 22:6n-3 follows the route 18:3n-3 $\xrightarrow{1}$ 18:4n-3 → 20:4n-3 $\xrightarrow{2}$ 20:5n-3 → 22:5n-3 $\xrightarrow{3}$ 22:6n-3. Similarly, the synthesis of 22:5n-6 follows the route 18:2n-6 $\xrightarrow{1}$ 18:3n-6 → 20:3n-6 $\xrightarrow{2}$ 20:4n-6 → 22:4n-6 $\xrightarrow{3}$ 22:5n-6. Steps 1, 2 and 3 are catalyzed by Δ_6, Δ_5 and Δ_4 desaturase systems, respectively, the other reactions being elongations. Much more is known on the regulation of Δ_6 and Δ_5 than of Δ_4 desaturases (20), but there is strong evidence that the desaturations rather than the elongations are rate-limiting steps in the sequences (21,22).

[1-^{14}C]20:5n-3 is readily transformed into [^{14}C]22:5n-3 and this into [^{14}C]22:6n-3 in the retina in vivo a few minutes after the injection of the precursor into the eye (23). This indicates that the retina does not rely on other organs (e.g., the liver) for the supply of 22:6, but is able to synthesize its own 22:6 from other (n-3) fatty acids (which, of course, must be available). The levels of n-3 pentaenes in retina lipids were much less affected by aging than n-3 hexaenes (Table 4), indicating that there was no defect in the availability of essential n-3 fatty acid precursors like 18:3n-3. Moreover, there is an important difference between the effect of aging shown here and that of essential fatty acid deficiency (24). Thus, during 18:2n-6 deficiency, 20:4n-6 is decreased in tissue lipids, but replaced by 20:3n-9 (made from oleate). During 18:3n-3 deficiency, 22:6n-3 decreases in lipids, being replaced by 22:5n-6 (made from linoleate). None of these compensatory mechanisms was observed in the aged retina. Moreover, the retina is known for the tenacity with which it holds 22:6 in its lipids, since generations of n-3 fatty acid precursor–deficient diets must elapse before a significant decrease of 22:6 can be observed in retina, as opposed to the rapid "adaptation" of liver, kidney and brain (25, 26). When the deficiency is eventually achieved, however (after at least two generations), the decreased 22:6n-3 is quantitatively replaced by 22:5n-6 (26–28). This is not the case with the aged retina, where both 22:6n-3 and 22:5n-6 decrease (Tables 2 and 3). It is quite coincidental that the two fatty acids whose synthesis is catalyzed by Δ_4 desaturase are the ones that undergo the most significant and consistent decreases, as illustrated in Table 8 by the ratios between the amounts (in nmol/mg protein) present in aged vs control retinas, as calculated from the data in Tables 1 and 2. This strongly suggests that, rather than a decreased availability of 18:3n-3 (and 18:2n-6) in the retina, an impairment of the Δ_4 desaturase system is probably responsible for the decreased levels of 22:6n-3 (and 22:5n-6) observed in retina lipids as a consequence of aging.

It is noteworthy that, in addition to 22:6n-3, other n-3 hexaenoic fatty acids are also decreased in aged retinas. This is clearly evident for PS (24:6n-3, Table 2) and for PC (Table 3), which contains a whole series of polyenoic fatty acids ranging from 24 to 36 carbons. The very long chain polyenes of PC fit into the general tendency observed

TABLE 8

Ratios Between Amounts (in nmol/mg Protein) of Fatty Acids in Aged vs Control Retinas (Calculated from Data in Tables 1 and 2)

	PC	EGP	PS	PI
20:4n-6	0.9	0.9	1.0	0.8
22:4n-6	1.0	0.7	0.6	1.0
22:5n-6	0.4	0.3	0.2	<0.1
20:5n-3	4.1	1.5	0.8	1.5
22:5n-3	1.2	1.1	0.9	>1.6
22:6n-3	0.6	0.7	0.7	0.9

PC, phosphatidylcholine; EGP, ethanolamine glycerophospholipids; PS, phosphatidylserine; PI, phosphatidylinositol.

for other polyenes, namely, that n-3 hexaenes (as 22:6n-3) decrease, and that they do so to a larger extent than n-3 pentaenes (as 20:5 and 22:5n-3) and n-6 tetraenes (as 20:4 or 22:4n-6). Very long chain tetraenes of vertebrate retina belong to the n-6 series and hexaenes to the n-3 series, and there are very long chain pentaenes from both the n-3 and n-6 families (2,3). The latter are in negligible amounts in rat retina but occur in larger proportions in rabbit and chicken retina PC (2). In chickens, it was observed that very long chain (n-6) pentaenes increase while very long chain (n-3) hexaenes decrease under dietary conditions that lead to an increased 22:5n-6/22:6n-3 ratio (2). The concomitant decrease of very long chain hexaenes and 22:6 observed here in rat retina PC is consistent with the idea that less 22:6 is produced during aging, since the impact is felt in the "shortest" hexaene as well as in the subsequently longer hexaenes that are synthesized by successive elongations of 22:6 (Rotstein, N.P., and Aveldaño, M.I., unpublished work). This fits into what is known on the synthesis of polyenoic fatty acids, i.e., that the rate-limiting steps are those catalyzed by desaturases, rather than those involving elongations, which mainly depend on the availability of the respective precursors (21), in this case 22:6. If the decrease in polyenes were exclusively due to increased rates of peroxidation during aging, one would probably not observe such trends of selectivity toward hexaenes and, especially, there would be no apparent reason for a decrease in 22:5n-6 much larger than that of 22:5n-3, since both fatty acids have identical number of carbons and double bonds.

It is evident from the [^{14}C]22:6 incorporation experiments that if docosahexaenoate is available, it is efficiently taken up by the aged retina, and it is most actively introduced into the phospholipid classes that show the largest decreases, not only of 22:6n-3 but of longer n-3 hexaenes. These observations are interesting from a functional point of view, since there is evidence that n-3 polyunsaturated fatty acids are necessary for the normal electrical response in visual excitation. Thus, a decrease of retina 22:6n-3 attained by dietary manipulation results in a change of the component of the electroretinogram that is generated by photoreceptors (27). Docosahexaenoate (27) as well as very long chain polyenoic fatty acids (2) are highly concentrated in the lipids of photoreceptor membranes. Therefore, a decrease in these fatty acids, which is observed even when the lipids of the entire retina are analyzed, must play an important role among the causes of vision impairment, which almost invariably accompanies senescence. The avidness with which aged retinas incorporate [^{14}C]22:6 in their 22:6-depleted lipids suggests the exciting possibility that an adequate supply of this fatty acid in the diet (rather than of its precursors) might help prevent, or at least delay, one of the many biochemical alterations that set in with aging.

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Calmodulin Antagonists Suppress Cholesterol Synthesis by Inhibiting Sterol Δ^{24} Reductase

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Preincubation of hepatoma cells and human skin fibroblasts in the presence of the calmodulin antagonists trifluoperazine and N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide resulted in a dose-dependent suppression of [^{14}C]mevalonolactone incorporation into cholesterol. At a calmodulin antagonist concentration of 25 μmol, the incorporation of [^{14}C]mevalonolactone into cellular cholesterol was suppressed to about 30% (hepatoma cells) and 10% (human skin fibroblasts) of control values. When the total nonsaponifiable [^{14}C]lipids were separated and analyzed by two-dimensional thin layer chromatography, an accumulation of [^{14}C]desmosterol was observed along with reduced formation of [^{14}C]cholesterol. However, when cells were preincubated in the presence of [^{14}C]dihydrolanosterol, [^{14}C]cholesterol formation was not inhibited by the calmodulin antagonists. About 25% of the cell-associated dihydrolanosterol radioactivity was converted to cholesterol in both control and calmodulin antagonist-pretreated cells. The data suggest that calmodulin antagonists prevent the conversion of desmosterol into cholesterol by inhibiting sterol Δ^{24} reductase and that the enzymes catalyzing sterol ring modifications are not affected by the inhibitors.
Lipids 22, 261-265 (1987).

Inhibitors of cholesterol biosynthesis are valuable tools for studying the regulation of cholesterol metabolism in cultured cells. Compactin (1), 3β-[2-(diethylamino)ethoxyl]-androst-5-en-17-one hydrochloride (U18666 A) (2,3), 4,4,10β-trimethyl-*trans*-decal-3β-ol (TMD) (4) and 1-[p-(β-diethylaminoethoxy)-phenyl]-1-(p-tolyl)-2-(p-chlorophenyl)-ethanol (MER 29, Triparanol) (5) have been found to inhibit cholesterol biosynthesis by acting at the level of 3-hydroxy-methylglutaryl coenzyme A (HMG CoA; EC 1.1.1.34) reductase (1-3), 2,3-oxidosqualene cyclase (EC 5.4.99.7) (2,4) and sterol Δ^{24} reductase (desmosterol reductase, EC 1.3.1.?) (5), respectively. The results obtained with these inhibitors suggest that oxysterols derived from squalene 2,3:22,23 dioxide may also act as physiological regulators of HMG CoA reductase (3).

Recently, the specific calmodulin antagonists N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) and trifluoperazine have been shown to suppress the cholesterol synthesis in human skin fibroblasts. A concomitant stimulation of low density lipoprotein receptor synthesis was found to be an independent effect (6). The present investigation describes the inhibitory effect of the calmodulin antagonists W-7 and trifluoperazine on the cholesterol synthesis in hepatoma cells and human skin fibroblasts. An analysis of the intermediates of cholesterol synthesis accumulated under the influence of calmodulin antagonists gives evidence for a preferential inhibition of the conversion of desmosterol to cholesterol by blocking sterol Δ^{24} reductase.

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MATERIALS AND METHODS

W-7 was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). Trifluoperazine was purchased from Sigma (Taufkirchen, Federal Republic of Germany). 25-Hydroxycholesterol was obtained from Steraloids Inc. (Wilton, New Hampshire). Lanosterol, cholesterol, squalene and desmosterol were products of Sigma. Lanosterol was purified from contaminating dihydrolanosterol according to ref. 8. [2-^{14}C]Mevalonic acid lactone and [26,27-^{3}H]25-hydroxycholesterol were purchased from NEN (Dreieich, Federal Republic of Germany). All other chemicals and organic solvents were from reputable sources and of analytical grade.

Squalene-2,3-oxide and squalene-2,3:22,23-dioxide were synthesized according to Chang et al. (4) and Field and Holmlund (7). [^{14}C]Dihydrolanosterol was synthesized enzymatically from [^{14}C]mevalonolactone according to the procedure described by Gibbons and Mitropoulos (8). Products of synthesis were acetylated and purified by thin layer chromatography (TLC) on silver nitrate-impregnated silica gel plates and visualized by radiofluorography. Acetyldihydrolanosterol was saponified, and the resulting dihydrolanosterol was extracted with hexane and purified by TLC. [^{3}H]Desmosterol was synthesized from [^{3}H]25-hydroxycholesterol according to the method of Svoboda and Thompson (9).

Cells. Human fibroblasts from skin explants of healthy donors were cultured as described elsewhere (6). Hepatoma cell line HepG2 was a gift of A. Schwarz, Department of Pediatrics, Harvard University (Boston, Massachusetts). Cells were grown in minimal essential medium supplemented with essential amino acids and 10-20% fetal calf serum. All experiments were performed with cells grown to confluency (2.5-3.0 × 10^{6} cells/25 cm^{2} flask).

Incubation conditions. Cells were preincubated for 24 hr in lipoprotein-deficient medium and 12 hr in the presence of the calmodulin antagonists prior to the addition of the radioactive metabolic precursors [^{14}C]mevalonolactone or [^{14}C]dihydrolanosterol in absolute alcohol (0.1% by vol). The incubation was stopped by extensive washing of cell layers with ice-cold buffered saline. Cells were scraped from the culture vessels with a rubber policeman and harvested by centrifugation.

Total lipids were obtained by three cycles of vortexing the cells in chloroform/methanol (2:1, v/v), extracted for 8 hr and saponified at 80 C for 30 min with 1 M KOH in methanol/benzene (4:1, v/v). Water was added, and the nonsaponifiable lipids were extracted with hexane. The solvent was evaporated using a stream of N_2. Lipids were solubilized in small volumes of chloroform/methanol (2:1, v/v) and submitted to two-dimensional TLC as described by Sexton et al. (2). Radioactive lipid (-containing) spots were visualized by radiofluorography, scraped into vials containing scintillation fluid and counted for radioactivity. When individual lipid fractions were further analyzed by TLC, the radioactive spots were scraped into vials

containing chloroform/methanol (2:1, v/v) and extracted. Synthetic products were tentatively identified by cochromatography using known nonradioactive reference substances. The positions of the lipids were ascertained by radiofluorography followed by spraying the plates with concentrated sulfuric acid and incubation at 120 C. To establish unequivocally the identity of individual sterol fractions, the radioactive lipids were acetylated (8) and cochromatographed together with correspondingly acetylated reference substances on 10% silver nitrate-impregnated silica gel plates in hexane/benzene (50:50, v/v).

In vitro assay of sterol Δ^{24} reductase. Microsomes and postmicrosomal fractions were prepared from rat liver homogenates according to the method described by Dempsey (10). Microsomal pellets were washed twice with 0.1 M potassium phosphate buffer, pH 7.3, and recentrifuged for 30 min at 105,000 × g. Incubation mixtures contained 1 ml of microsomes (about 10 mg protein) suspended in 0.1 M potassium phosphate buffer, pH 8.3, 2.5 ml (18 mg/ml) of 105,000 × g supernatant and 85 × 10^3 cpm (1.5 μg) of [^{3}H]desmosterol in 150 μl of propylene glycol. Following the addition of 1 μl/ml of 25 mM trifluoperazine or W-7 in dimethylsulfoxide (1 μl/ml), the mixture was incubated for 15 min at 37 C. The enzymatic reaction was started by adding NADPH in a small volume of incubation buffer to a final concentration of 0.45 mM. The incubation was terminated after 2 hr by adding 15 ml chloroform/methanol (2:1, v/v) and extraction of lipids. The extraction procedure was repeated three times. The lipid extracts were pooled and the organic solvents were evaporated under vacuum. The total lipids were acetylated (8) and extracted in hexane, and aliquots were submitted to separation on 10% silver nitrate-impregnated silica gel plates in hexane/benzene (50:50, v/v). Radioactive spots were visualized by radiofluorography, scraped into scintillation fluid-containing vials and counted for radioactivity.

RESULTS

Calmodulin antagonists W-7 and trifluoperazine prevent conversion of desmosterol to cholesterol. Hepatoma cells incorporated ^{14}C radioactivity from [2-^{14}C]mevalonic acid lactone during a 6-hr pulse period preferentially into cholesterol. Intermediates of cholesterol synthesis (lanosterol, dihydrolanosterol and desmosterol) were detectable as minor components, which were, however, converted to cholesterol during an 18-hr chase period (Fig. 1A).

Preincubation of the cells in 25 μM W-7 or trifluoperazine led to an accumulation of [^{14}C]desmosterol (spot no. 6 of Figs. 1B and 1C). In addition, squalene 2,3-oxide and 2,3:22,23 squalene dioxide, an oxygenated by-product of sterol synthesis, accumulated within nonsaponifiable lipids. In a pulse-chase experiment, W-7 and trifluoperazine partially or almost completely prevented the conversion of desmosterol to cholesterol (Figs. 1B and 1C).

Desmosterol and cholesterol (spot numbers 6 and 5 of Fig. 1) were identified as acetyl derivatives. To do this, spots 6 and 5 were recovered from TLC plates, acetylated with acetic anhydride and cocrystallized with authentic acetyl derivatives of cholesterol and desmosterol in various solvent systems. In all systems, radioactive, acetylated material from the spots cochromatographed

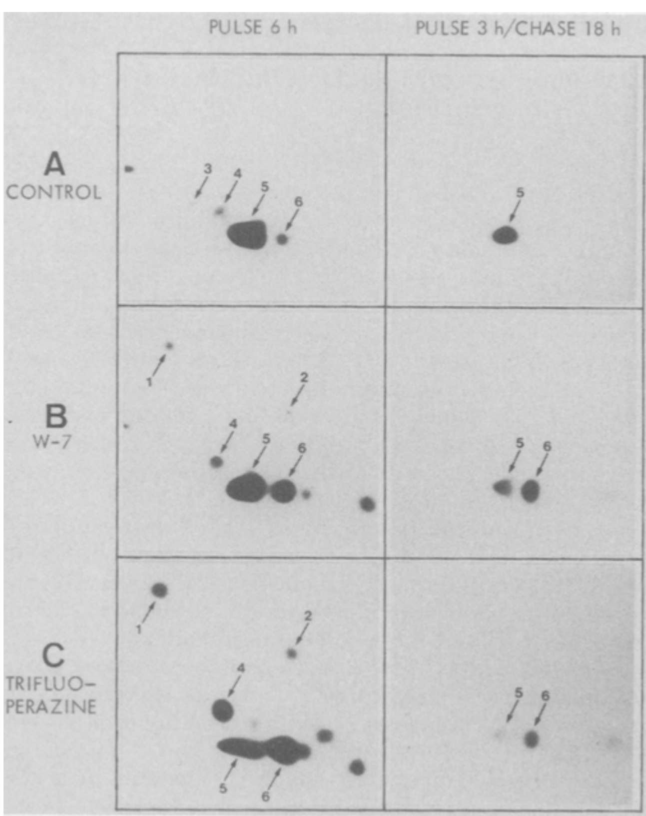


FIG. 1. Effect of N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) and trifluoperazine on sterol synthesis in HepG2 cells. Cells were preincubated for 12 hr in the presence of 25 μM W-7 or trifluoperazine prior to addition of 5 μCi/ml of [2-^{14}C]mevalonolactone, and the incubation continued for 6 hr. In pulse-chase experiments the incubation in the presence of the radioactivity was terminated after 3 hr by discarding the radioactive medium. Cell layers were washed three times with fresh nonradioactive medium and then maintained for 18 hr either in the presence or absence of the indicated concentrations of the calmodulin antagonists. Total lipids of harvested cells were extracted with chloroform/methanol (2:1, v/v) several times and processed as described in Materials and Methods. The nonsaponifiable lipids were submitted to separation on silica gel plates by two-dimensional thin layer chroamtography as described in Materials and Methods. Radioactive spots were identified by radiofluorography. Identified lipids are as follows: 1, squalene-2,3-oxide; 2, squalene-2,3:22,23-dioxide; 3, dihydrolanosterol; 4, lanosterol; 5, cholesterol; 6, desmosterol.

with reference acetyl derivatives. Figure 2 shows fluorograms of [^{14}C]acetylcholesterol and [^{14}C]acetyldesmosterol, which were found in positions virtually identical to those of the nonradioactive reference substances made visible with concentrated sulfphuric acid (not shown).

Quantitative data on the effect of W-7 and trifluoperazine on cholesterol synthesis in hepatoma cells and skin fibroblasts are given in Table 1. While the synthesis of total nonsaponifiable lipids in hepatoma cells was suppressed by calmodulin antagonists to only 75-80% of control values, the ratio of cholesterol to desmosterol decreased from 29.6 (control) to 0.54 (W-7) and 0.33 (trifluoperazine) under the influence of these agents. As demonstrated in Figure 3, the inhibition of cholesterol synthesis by trifluoperazine is dose-dependent.

Analogous results were obtained for human skin fibroblasts (Table 1). The ^{14}C radioactivity incorporated into

TABLE 1

Effect of the Calmodulin Antagonists W-7 and Trifluoperazine on Cholesterol Synthesis from [^{14}C]Mevalonolactone in HepG2 Cells and Human Skin Fibroblasts

		HepG2 cells: [2-^{14}C]mevalonolactone incorporation (cpm × 10^{-3}/mg cell protein)			Fibroblasts: [2-^{14}C]mevalonolactone incorporation (cpm × 10^{-3}/mg cell protein)		
Additions	Final concentration (μM)	Total nonsaponifiable lipids	Desmosterol	Cholesterol	Total nonsaponifiable lipids	Desmosterol	Cholesterol
None	—	9.6	0.3	8.9	3.7	—	3.4
W-7	25	7.8	4.6	2.5	3.4	3.1	0.2
Trifluoperazine	25	7.2	5.1	1.7	3.1	2.7	0.1

W-7, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide. Cells grown to confluency were preincubated for 24 hr in lipoprotein-deficient medium and 12 hr in the presence of the indicated concentrations of the calmodulin antagonists prior to the addition of 5 μCi/ml (HepG2 cells) and 10 μCi/ml (fibroblasts) of [2-^{14}C]mevalonic acid lactone. Incubation with the radioactive precursor was terminated after 6 hr by removing the medium and by extensive washing of the cell layers with Hank's solution. Fresh lipoprotein-deficient medium was then added, and the cells were scraped off the culture vessels and collected by centrifugation 18 hr later. Extraction, saponification and separation of radioactive sterols were as described in Materials and Methods. Values are means of two experiments made in duplicate.

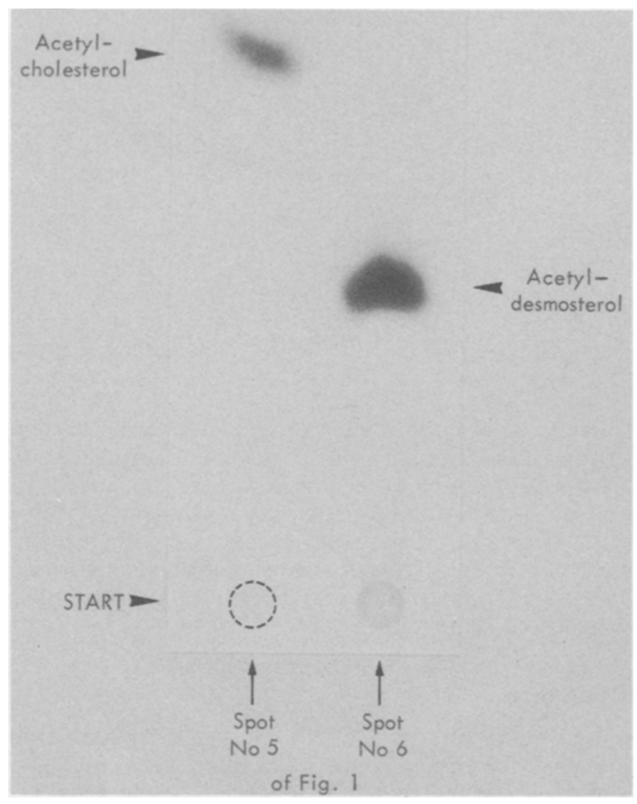


FIG. 2. Identification of synthetic products accumulated in HepG2 cells during a pulse-chase experiment in the presence of trifluoperazine. Radioactive spots designated 5 and 6 in Fig. 1 from pulse-chased and trifluoperazine-pretreated cells were scraped off into vials containing chloroform/methanol (2:1, v/v). The extracted lipids were acetylated and chromatographed on silver nitrate–impregnated silica gel G plates (Materials and Methods). Nonradioactive acetylcholesterol and acetyldesmosterol were cochromatographed. The positions of lipids on developed plates were determined by radiofluorography and by spraying the plates with concentrated sulphuric acid.

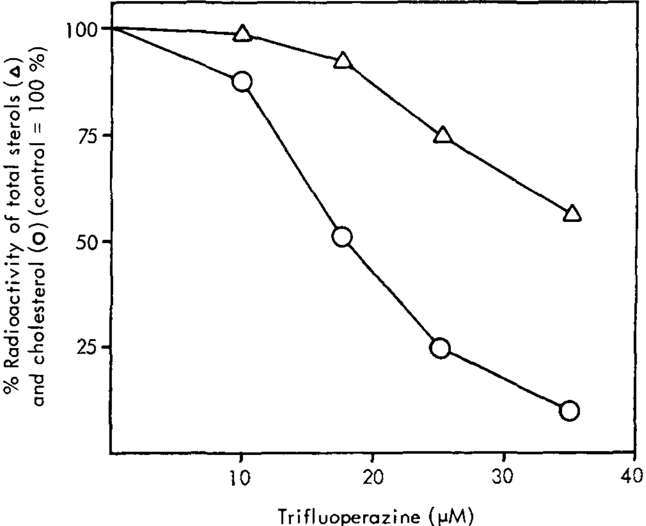


FIG. 3. Inhibition of sterol synthesis in HepG2 cells relative to trifluoperazine concentration. Cells were preincubated for 24 hr in lipoprotein-deficient medium and 12 hr in the presence of the indicated concentrations of trifluoperazine. Following incubation for 3 hr with 5 μCi/ml [2-^{14}C]mevalonolactone, the lipids were extracted from the cells as described in Materials and Methods and Fig. 1. Lipids were separated by two-dimensional thin layer chromatography. Total sterol synthesis was estimated by counting an aliquot of nonsaponifiable lipids.

the total nonsaponifiable lipids of fibroblasts was two to three times less than that incorporated into the lipids of hepatoma cells. Furthermore, the shift in the ratio of cholesterol to desmosterol under the influence of calmodulin antagonists was remarkable; the formation of [^{14}C]cholesterol dropped from 100% (control) to 3–6% (calmodulin antagonists), which caused desmosterol to accumulate.

Calmodulin antagonists inhibit sterol Δ24 reductase. The conversion of desmosterol to cholesterol is catalyzed by sterol Δ24 reductase. This enzyme has been reported to be present in various cholesterol-producing tissues and to reside in the microsomal fraction (11), but has not yet been characterized in detail. Rat liver microsomes were used to study the effect of W-7 and trifluoperazine on sterol Δ24 reductase activity in vitro. The data given in Table 2 demonstrate that in control experiments about 60% of the [^{3}H]desmosterol is converted within 2 hr to

TABLE 2

In Vitro Effect of W-7 and Trifluoperazine on Sterol Δ²⁴ Reductase of Isolated Rat Liver Microsomes

Additions	μM	^{3}H radioactivity Desmosterol (cpm)	Cholesterol (cpm)	% of Substrate converted
None	—	11240	18760	62.5
W-7	25	24150	5980	19.8
Trifluoperazine	25	25860	5320	17.0

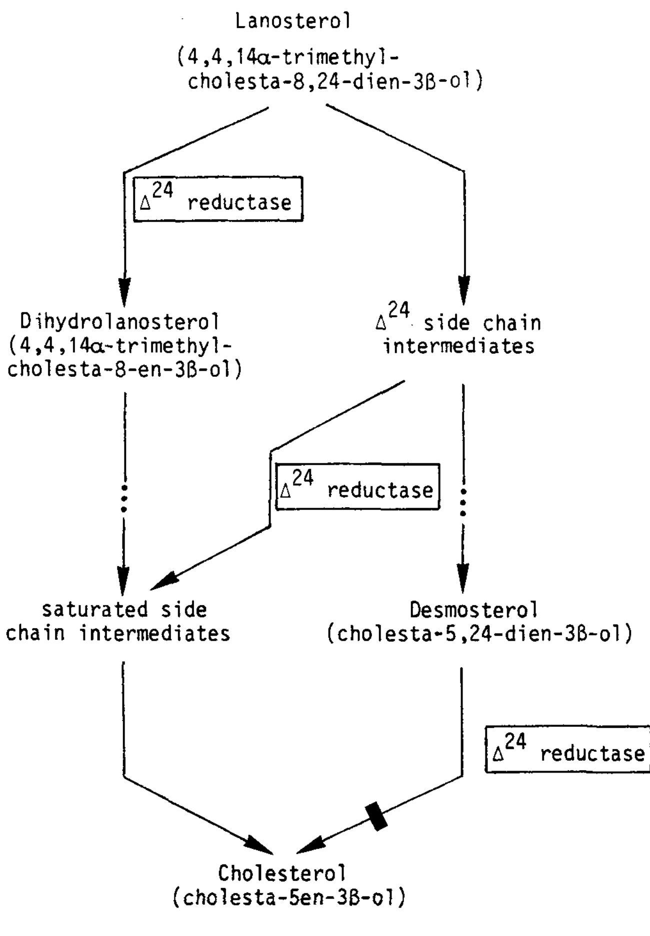
W-7, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide. [^{14}C]-Desmosterol (1.5 μg ≈ 85 × 10^3 cpm/assay) was used as substrate. Incubation conditions, lipid extraction, separation and acetylation are described in Materials and Methods. Comparable amounts of radioactivity (about 30,000 cpm) of acetylated total [^{3}H]lipids were submitted to thin layer chromatography. All experiments were performed in duplicate.

TABLE 3

Effect of W-7 and Trifluoperazine on Cholesterol Synthesis from [^{14}C]Dihydrolanosterol in HepG2 Cells

Additions	Final concentration (μM)	^{14}C radioactivity Dihydrolanosterol (cpm = 10^3)	Cholesterol (cpm × 10^3)
None	—	36.1	9.1
W-7	25	35.3	7.5
Trifluoperazine	25	37.2	6.9

W-7, N-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide. Cells were preincubated for 24 hr in lipoprotein-deficient medium and 12 hr in the presence of 25 μM W-7 or trifluoperazine. Then [^{14}C]dihydrolanosterol (2.5 × 10^5 cpm/ml) in absolute alcohol (10 μl/ml) was added, and incubation was continued for 6 hr. After extensive washing with buffered saline the cells were scraped from the culture vessels and harvested by centrifugation. Lipids were extracted, and aliquots with equal amounts of radioactivity were submitted to separation on paraffin oil-impregnated silica gel G plates using acetone/water saturated with paraffin (85:15, v/v) as solvent. Radioactive lipids were detected by fluorography, recovered and counted. The identity of lipid fractions was ascertained by comparison with cochromatographed nonradioactive dihydrolanosterol and cholesterol. Values are means of two experiments made in duplicate.



SCHEME 1

[^{3}H]cholesterol by rat liver microsomes in the presence of NADPH. Under these conditions, W-7 and trifluoperazine significantly inhibited the enzymatic reduction of desmosterol to cholesterol (30% of control values).

Enzymatic conversion of dihydrolanosterol to cholesterol is not inhibited by calmodulin antagonists. The enzymatic conversion of lanosterol to cholesterol is comprised of several reactions: reduction of the Δ²⁴ double bond, removal of three methyl groups and shift of the nuclear double bond from the Δ⁸ to the Δ⁵ position. Calmodulin antagonists inhibit predominantly the reduction of the Δ²⁴ double bond, because when cells were incubated in the presence of [^{14}C]dihydrolanosterol, nearly equal amounts of ^{14}C radioactivity were incorporated into cholesterol in both control and calmodulin antagonist-pretreated HepG2 cells (Table 3). Dihydrolanosterol has been found to be a sterol intermediate in the biosynthesis of cholesterol and to be convertible to cholesterol (12,13). However, the reaction sequence dihydrolanosterol → cholesterol seems to be bypassed in HepG2 cells; only 25% of the internalized [^{14}C]dihydrolanosterol was converted to cholesterol by these cells.

DISCUSSION

The present study indicates that the calmodulin antagonists W-7 and trifluoperazine suppress the biosynthesis of cholesterol by blocking sterol Δ²⁴ reductase. The enzymatic reduction of the Δ²⁴ double bond is one of the necessary reactions during cholesterol synthesis. Reduction of the bond may occur at any of several points during transformation of lanosterol to cholesterol. Thus, sterol Δ²⁴ reductase may catalyze an initial conversion of lanosterol to 24,25-dihydrolanosterol, which has been found to be convertible to cholesterol (13). An alternative metabolic pathway leads via a series of Δ²⁴ side chain intermediates from lanosterol to desmosterol, which is finally converted to cholesterol by sterol Δ²⁴ reductase (see Scheme 1). The finding that the calmodulin antagonists used in this study do not inhibit the conversion of [^{14}C]24,25-dihydrolanosterol to cholesterol (Table 3), but cause an accumulation of desmosterol (Table 1), suggest

a) that calmodulin antagonist-induced inhibition of sterol Δ^{24} reductase is relatively specific and b) that the enzymes catalyzing sterol ring modifications are not affected by the inhibitors.

Calmodulin antagonists are reported to be potent and specific inhibitors of calmodulin-mediated reactions and calmodulin-dependent enzyme activities (14). Such inhibition appears to result from conformational restrictions conferred upon calmodulin by the bond antagonist (15). Our results, however, do not allow any conclusion as to whether sterol Δ^{24} reductase is a calcium calmodulin-regulated enzyme. In this regard, the conversion of [^{3}H]-desmosterol to [^{3}H]cholesterol by isolated rat liver microsomes under in vitro conditions was found to be inhibited by the calmodulin antagonists (Table 2), but the calcium calmodulin concentration of the microsomal fraction was not determined. Furthermore, no information is available to indicate whether the activity of sterol Δ^{24} reductase is controlled by calcium calmodulin-dependent protein-kinases or by phosphoprotein phosphatases. In view of the previously reported inhibition of sterol Δ^{24} reductase by U18666 A (2,3). TMD (4) and triparanol (16), the enzyme seems to be highly sensitive to agents with disparate chemical structure.

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Liver Parenchymal Cells Differ from the Fat-storing Cells in Their Lipid Composition

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The neutral lipid and phospholipid compositions of purified sinusoidal (fat-storing, endothelial and Kupffer) cells, parenchymal cells and liver homogenates were determined by thin layer chromatography. In addition, the retinoid content of the same purified cell populations was determined by high performance liquid chromatography. From each cell type, both a lipid droplet fraction and a pellet fraction (containing the majority of the remaining cell organelles) were prepared by differential centrifugation. Electron microscopic analysis showed that lipid droplets isolated from fat-storing cells were larger (up to 8 μm) than those isolated from parenchymal cells (up to 2.5 μm). Moreover, the parenchymal lipid droplets seemed to be surrounded by a membranous structure, while the fat-storing lipid droplets seemed not to be. Both fat-storing and parenchymal cells contained high concentrations of neutral lipids, 57.9 μg and 71.0 μg/10^6 cells, respectively, while endothelial and Kupffer cells contained only 8.6 μg and 13.8 μg/10^6 cells of neutral lipids, respectively. Sixty-five percent of fat-storing cell lipid droplet fractions comprised esters of retinol and cholesterol. This combined ester fraction contained mainly retinyl esters. In addition, considerable quantities (20%) of triglycerides were present. Parenchymal cell lipid droplet fractions comprised triglycerides (62%) and cholesteryl esters (up to 30%). The pellet fractions prepared from all four cell types consisted mainly of cholesterol (41-67%) and free fatty acids (20-28%). The phospholipid content was much higher in parenchymal cells than in the sinusoidal liver cell types. The relative proportions of the four major phospholipid classes were comparable in all liver cell types analyzed. It is concluded that parenchymal cell lipid droplets comprised mainly triglycerides and cholesteryl esters, which is in agreement with the function of parenchymal cells in lipid metabolism. Fat-storing cell lipid droplets consisted of retinyl esters and triglycerides, which correlates well with their function in retinoid storage and metabolism.
Lipids 22, 266-273 (1987).

Lipids found in liver cells are located mainly in membranes or in lipid droplets. Liver lipid droplets are almost exclusively observed in two different liver cell types, parenchymal and fat-storing cells. Fat-storing cells contribute considerably to the total lipid droplet volume in the liver (1,2).

It is well established that liver parenchymal cells have a function in triglyceride and cholesterol synthesis and metabolism (3). Fat-storing cells, on the other hand, have been shown to be important in retinoid storage (4,5) and metabolism (6). It is unknown whether these different functions in lipid metabolism are reflected in the cellular lipid composition. Limited information is available on some details of the lipid content of two of the liver cell types (7,8). In this study, neutral lipids and phospholipids present in a total liver homogenate and in isolated parenchymal and fat-storing cells, as well as in two other major liver cell populations, Kupffer and endothelial, were determined. Neutral lipids were also determined in the lipid droplets prepared from these cell types.

MATERIALS AND METHODS

Animals. Female Brown Norway/Billingham Rijswijk (BN/BiRij) rats aged 12 mo and weighing 180 ± 10 g were used. All rats were fed standard laboratory chow ad libitum (Diet AM II, Hope Farms, Woerden, The Netherlands) and were not fasted before the experiment. The chow contained 5.5 mg retinyl acetate and 72 g fat per kg diet, of which 17 g was derived from animal fats. Specific pathogen-free derived rats were maintained under "clean conventional conditions" (9). The status of this strain of rats has previously been characterized with regard to liver vitamin A status and serum concentrations of triglycerides, cholesteryl esters and retinoids (4).

Chemicals and solvents. All chemicals and solvents were of reagent grade or high performance liquid chromatography (HPLC) grade, if required. The solvents of reagent grade used for the extraction and chromatography of lipids were distilled twice to exclude any fatty contamination. Vitamins D_2, D_3 and E, cholestenon, cholestane-3β,6α-diol, cholesterol, cholestane-3β,5α,6β-triol and bovine serum albumin were purchased from Sigma (St. Louis, Missouri).

Isolation of liver cells and lipid droplets. Nonparenchymal cell suspensions were prepared by perfusion and incubation of the liver with pronase and collagenase as described previously (10). Kupffer, endothelial and fat-storing cells were purified by density centrifugation (4) and centrifugal elutriation (11). Parenchymal cell suspensions were prepared at 37 C by perfusion and incubation of the liver with collagenase (4) and were further purified by centrifugal elutriation (4).

Lipid droplet fractions were prepared from the various isolated cell preparations by freeze-thawing followed by ultrasonification using an MSE 150 Watt Ultrasonic disintegrator. Sonification was continued until more than 95% of the cells were homogenized, as judged by light microscopy. Cell homogenates were centrifuged in a 0.25 M sucrose solution for 7.9×10^6 $g_{1/2}$.min. The top layer (containing the lipid droplets) was separated from the pellet by cutting the centrifuge tube in a tube-slicing apparatus.

Cell counts were performed for the yield of the cell fraction. The purity of the cell preparations and the lipid droplet fractions was determined by electron microscopy (12). Cells were fixed for electron microscopy using a 2% glutaraldehyde solution in 0.15 M cacodylate buffer (pH 7.4) (6), while lipid droplets were fixed using a freshly mixed 0.7% glutaraldehyde and 1% osmium tetroxide solution in 0.15 M cacodylate buffer (pH 7.4) and processed for electron microscopy as for isolated cells.

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Protein content of the isolated cell preparations was determined by the method of Lowry et al. (13) with bovine serum albumin as a standard.

The total liver homogenate was prepared from a liver perfused in situ with Gey's balanced salt solution for several minutes at a flow rate of 10 ml/min. After perfusion, the liver was weighed and cut into small pieces of about 1 mm^3 with razor blades.

Analysis of lipids and retinoids. Lipids were extracted from liver homogenates, cell preparations and isolated lipid droplets as described by Bligh and Dyer (14). Neutral lipids were separated using thin layer chromatography (TLC). Internal standards cholestane-3β,6α-diol and cholestane-3β,5α,6β-triol were added to allow the quantitation of neutral lipids and phospholipids, respectively. Lipid extracts were spotted on glass microscope slides coated with a layer of ±0.1 mm of Silica DO (Camag, Switzerland) in a thin line at a concentration of 2 mg/ml in chloroform. The slides were developed in small chromatography cylinders (height 100 mm, diameter 45 mm).

Neutral lipids were chromatographed using chloroform/methanol (95:5, v/v) up to half of the height of the slide. The slides were then dried and lipids were further developed using hexane/chloroform (60:25, v/v) up to the top of the slide (15).

Phospholipids were separated on the same type of slides, developed with methanol/chloroform/water (75:25:4, v/v/v) up to the top of the slide and dried. The slides were then developed for a further 10 min in the same solution and dried again.

Both neutral lipids and phospholipids were visualized by wetting the slides with sulphuric acid (10%, v/v) and charring by a two-step heating procedure. The first heating at 100 C allows the identification of cholesterol and cholesteryl esters by the red color of their bands. Retinyl esters were already charred at 100 C. The second heating at 200 C for 15 min charred all lipids and resulted in black bands. The intensity of the charred lipids was quantified after the second heating by photodensitometry and the use of a Shimadzu data module (CS 910). As the number of densitometric units per μg lipid is dependent on the percentage of carbon in a lipid class, densitometric values of each lipid class were multiplied by a correction factor (15). Correction factors for cholesterol and retinol were the same as were those for cholesteryl esters and retinyl esters.

Equivalent volumes of cell-free medium were run as blanks and subtracted as background values. Background values never made up more than 2% of the total amount of lipid detected. Free retinol and free cholesterol cochromatographed in the TLC system. Retinyl esters and cholesteryl esters could not be calculated as separate classes using densitometry due to an incomplete separation. The two-step heating procedure did, however, allow the identification of these two lipid classes. Moreover, the major constituent present within each specific band (either as retinyl esters or as cholesteryl esters) could be estimated because retinyl esters were quantified separately in each cell preparation by HPLC (see below). Phosphatidylserine and phosphatidylinositol cochromatographed with phosphatidylcholine.

Peak identification of both neutral and phospholipids was achieved using normal rat serum as a standard in a parallel run. All materials that would be in contact with the extracted lipids were rinsed twice before use with distilled chloroform to wash off contaminating lipids.

Retinoids, including retinol, retinyl oleate, retinyl palmitate and retinyl stearate, were analyzed and quantified by reverse phase HPLC using retinyl acetate as an internal standard as described previously (4). Retinoids were analyzed in liver homogenates and cell preparations after lyophilization and overnight extraction at 4 C with methanol/diisopropylether/water (31.7:63.3:5, v/v/v) containing 50 mM ethylenediaminetetraacetic acid and butylated hydroxytoluene (50 μg/ml) (4).

The extraction procedures for TLC (14) and HPLC (4) were evaluated by quantitating the lipid classes by TLC. The quantities of neutral lipids determined in a liver homogenate were significantly higher when the extraction method described by Bligh and Dyer (14) was used (25.0 ± 2.6 mg/g wet weight [n = 4] and 12.1 ± 1.0 mg/g wet weight [n = 2] for TLC and HPLC extraction procedures, respectively). The only exception was the combined retinyl ester/cholesteryl ester fraction, which was equally well extracted in the two extraction procedures (2.6 ± 0.0 mg/g wet weight [n = 2] and 2.5 ± 0.2 mg/g wet weight [n = 4], respectively).

Extraction and separation of lipids and retinoids were performed under an atmosphere of nitrogen to prevent lipid oxidation. Since retinoids are photosensitive, all procedures were performed in reduced (red) light or, when possible, in the dark.

RESULTS

Characteristics of isolated cells and lipid droplets. Isolated parenchymal, Kupffer, endothelial and fat-storing cells were obtained with good viability (80–90%). The purity of all cell preparations was determined by electron microscopy; the average purity varied from 72% for fat-storing cells to 98% for parenchymal cells (Table 1).

Lipid droplets were isolated from each cell preparation. Representative micrographs of lipid droplets from fat-storing cells and parenchymal cells are shown in Figures 1a and 1b. Lipid droplets isolated from parenchymal cells had diameters up to 2.5 μm. Lipid droplets from fat-storing cells tended to be larger than parenchymal cell lipid droplets; their sizes varied up to 8 μm. Isolated lipid droplets from parenchymal cells seemed to be surrounded by a limiting membrane (Fig. 1b, inset). Fat-storing cell lipid droplets were surrounded by a thin thread of cytoplasm, but not by a limiting membrane (Fig. 1a, inset).

TABLE 1

Characterization of Cell Preparations Used for Lipid Analysis[a]

Cell fraction	n	Purity (%)	Protein content (μg/10^6 cells)	Total retinoids (μg/10^6 cells)
Fat-storing cells	5	72 ± 7	96 ± 7	85.6 ± 14.8
Endothelial cells	5	80 ± 3	50 ± 4	2.2 ± 1.2
Kupffer cells	5	80 ± 2	106 ± 6	1.1 ± 0.4
Parenchymal cells	3	98 ± 1	1267 ± 280	2.2 ± 1.2

[a]Values represent the mean ± SEM.

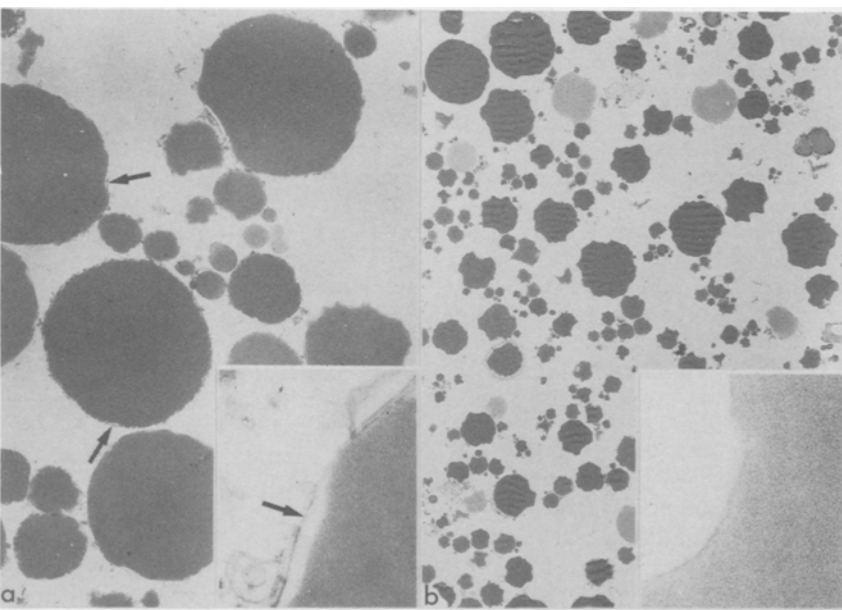

FIG. 1. Transmission electron micrograph of representative lipid droplet fractions. (a) Fat-storing cell-derived lipid droplet fraction. Lipid droplets of homogenous density and of variable diameter are shown (3,925×). Lipid droplets are surrounded by a thin thread of cytoplasm (arrows) but not by a limiting membrane (inset, 41,420×). (b) Parenchymal cell-derived lipid droplet fraction. Lipid droplets of variable density and size are shown (3,925×). Lipid droplets seem to be surrounded by a unilamellar membrane (inset, 93,740×).

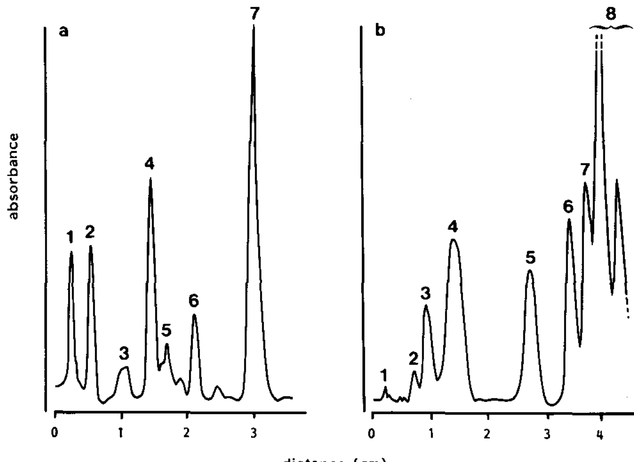

FIG. 2. TLC of extracts of fat-storing cell preparations. (a) Separation of neutral lipids. Peak identification: 1, sample origin containing phospholipids and cholestane-3β,5α,6β-triol; 2, cholestane-3β,6α-diol; 3, free fatty acids; 4, cholesterol/retinol; 5, ?; 6, triglycerides; 7, retinyl esters/cholesteryl esters. (b) Separation of phospholipids. Peak identification: 1, sample origin; 2, lysophosphatidylcholine; 3, sphingomyelin; 4, phosphatidylcholine, including phosphatidylserine and phosphatidylinositol; 5, phosphatidylethanolamine; 6, cholestane-3β,5α,6β-triol; 7, cholestane-3β,6α-diol; 8, neutral lipids.

All lipid droplet preparations were slightly contaminated with structures derived from mitochondria, lysosomes and ribosomes.

The yield and purity of the lipid droplet fractions derived from preparations of Kupffer and endothelial cells were low.

The pellet fractions of the cell lysates obtained after ultracentrifugation contained hardly any lipid droplets (results not shown). These pellets consisted mainly of cell organelles and a few intact cells.

Identification of lipid classes by TLC. Neutral lipids and phospholipids were separated using TLC. A representative chromatogram of neutral lipids present in a fat-storing cell preparation is shown in Figure 2a.

The main neutral lipid classes identified were free fatty acids, free cholesterol, triglycerides and esters of both cholesterol and retinol. Preparations of all isolated liver cell types, as well as the total liver homogenate, contained all these lipid classes. Vitamin E cochromatographed with the triglycerides, and vitamins D_2 and D_3 cochromatographed with cholesterol (not shown). The peaks that could be identified accounted for more than 80% of the total peak area, from which the phospholipids, carbohydrates and internal standards had been excluded. Other minor unidentified peaks were present, e.g., peak 5 (Fig. 2a). Peak 5 was detected in all cell fractions, but higher concentrations were found in fat-storing (Fig. 2a) and Kupffer cell preparations (not shown). In both cases, it accounted for 10–12% of the total peak area, again excluding phospholipids, carbohydrates and internal standards.

A representative chromatogram of the phospholipids present in isolated fat-storing cells is shown in Figure 2b. The main phospholipid classes identified in liver homogenates and in the four cell preparations were phosphatidylcholine, including phosphatidylserine and phosphatidylinositol, phosphatidylethanolamine, sphingomyelin and lysophosphatidylcholine. Together, these lipids accounted for more than 90% of the total phospholipids present.

TABLE 2

Concentrations of Neutral Lipids and Retinoids in Serum and Total Liver Homogenates of BN/BiRij Rats Determined by TLC and HPLC, Respectively[a]

		TLC					HPLC
Sample	n	Free fatty acids	Cholesterol/retinol	Triglycerides	Cholesteryl/retinyl esters	Total	Retinoids
Serum[b]	5	23.9 ± 1.9	25.5 ± 4.2	32.3 ± 6.2	107.9 ± 4.2	189.5 ± 9.8	0.017 ± 0.002
Liver[c]	4	8.1 ± 1.1	3.6 ± 0.1	10.9 ± 2.0	2.5 ± 0.2	25.0 ± 2.6	2.3 ± 0.4

[a]BN/BiRij, Brown Norway/Billingham Rijswijk; TLC, thin layer chromatography; HPLC, high performance liquid chromatography. Values represent the mean ± SEM.
[b]mg/100 ml.
[c]mg/g wet weight.

TABLE 3

Concentrations of Neutral Lipids in Isolated Fat-storing, Endothelial, Kupffer and Parenchymal Cells by TLC[a]

Cell fraction	n	Free fatty acids	Free cholesterol	Triglycerides	Retinyl/cholesteryl esters	Total neutral lipid
Fat-storing cells	5	6.0 ± 0.8[e,k]	10.2 ± 1.3[e]	11.0 ± 3.0[e,p]	30.7 ± 4.6[e,k,p]	57.9 ± 6.4[e,k]
Endothelial cells	5	1.0 ± 0.2[f]	4.7 ± 0.6[f,p]	1.3 ± 0.2[f,k,p]	1.6 ± 0.5[f,p]	8.6 ± 1.1[f,k,p]
Kupffer cells	5	1.3 ± 0.3[f]	6.3 ± 0.2[p]	3.9 ± 0.3[e,p]	2.3 ± 0.2[f,p]	13.8 ± 0.7[f,e,p]
Parenchymal cells	3	4.3 ± 2.1	16.9 ± 2.2[e,k]	43.7 ± 8.1[f,e,k]	6.1 ± 1.0[f,e,k]	71.0 ± 11.7[e,k]

[a]TLC, thin layer chromatography. Values are expressed as μg lipid per 10^6 cells and represent the mean ± SEM. Values differ significantly ($p < 0.01$) from values for *f*, fat-storing cells; *e*, endothelial cells; *k*, Kupffer cells; and *p*, parenchymal cells. Values were tested for significance using the unpaired *t*-test (two-tail).

Neutral lipid content of serum and total liver. Using the TLC method described above, the neutral lipid contents of serum and liver homogenate were determined to assess the lipid status of the rats used in this study (Table 2). Serum and total liver contained concentrations of triglycerides and free fatty acids, which were on the same order of magnitude as those previously reported in the literature (16–20; Table 2).

HPLC analysis of serum retinoids showed them to be almost exclusively retinol. Retinyl esters were not detectable or were present in very low concentrations. Consequently, the cholesteryl/retinyl ester fraction in serum detected by TLC consisted of cholesteryl esters only.

HPLC analysis of liver retinoids showed that retinol was present in a concentration of only 50.3 ± 1.9 μg/g wet weight (data not shown). The majority of the retinoids present in a liver homogenate were retinyl esters (97%; see Table 2). Therefore, a major contribution of retinol to the cholesterol/retinol peak detected by TLC could be ruled out. On the other hand, retinyl esters were a major part of the total cholesteryl/retinyl ester fraction of the liver.

Neutral lipid composition of isolated cell preparations. TLC analysis showed that parenchymal cells contained high amounts of neutral lipid per 10^6 cells, as did fat-storing cells (Table 3). Kupffer and endothelial cells, however, contained low amounts of neutral lipids. Kupffer cell fractions contained significantly more neutral lipid than endothelial cells. When calculated per mg cell protein (Tables 1 and 3), fat-storing cells contained significantly more neutral lipid (603 μg/mg protein) than parenchymal cells (56 μg/mg protein).

Free fatty acids were present in relatively high concentrations in both fat-storing cells and parenchymal cells as compared to Kupffer and endothelial cells (Table 3). In all cell types, free fatty acids accounted for only about 10% of the total neutral lipids present (Fig. 3). The percentage of free fatty acids present in liver homogenates (32%) was considerably higher than that in any of the isolated cell fractions (6–12%).

Very small amounts of retinol were present in fat-storing cell preparations (0.1 ± 0.0 μg/10^6 cells) as determined by HPLC. Thus, retinol did not make a major contribution to the cholesterol/retinol fraction.

The concentration of cholesterol, a major component of cell membranes, was significantly higher in the parenchymal cells (Table 3) than in the three other cell types. Fat-storing cells contained significantly more free cholesterol than did endothelial or Kupffer cells. Cholesterol made up a major part of the neutral lipids present in both endothelial cells and Kupffer cells (Fig. 3).

Parenchymal cells contained the highest concentration of triglycerides (Table 3). Triglycerides accounted for 62% of the total amount of neutral lipids present in the parenchymal cell fractions, which is higher than the percentages present in the other cell types (Fig. 3).

Esters of cholesterol and retinol were present in high concentrations in fat-storing cells (Table 3). These two classes of esters together accounted for most of the lipids in the fat-storing cells (51%; Fig. 3). The two-step heating procedure showed that the esters already charred at 100 C, indicating that the majority of these esters were retinoids, which was confirmed by HPLC analysis (Table 1).

The concentration of cholesteryl and retinyl esters was

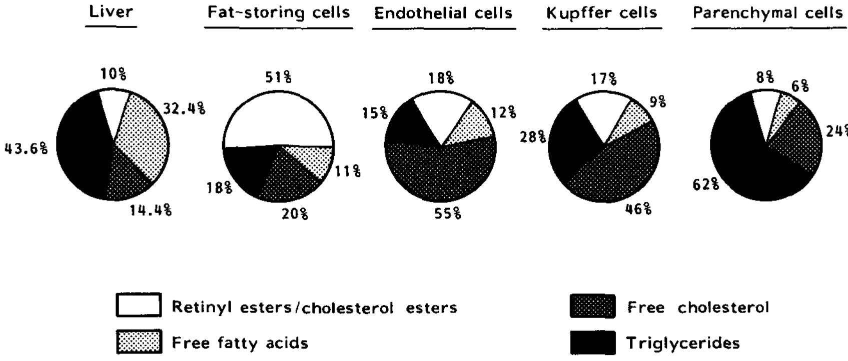

FIG. 3. Diagrams showing the neutral lipid composition of fat-storing, endothelial, Kupffer and parenchymal cells and liver homogenates, expressed as percentage of the total amount of neutral lipids determined. Neutral lipid classes identified and included are free fatty acids, cholesterol, triglycerides and esters of cholesterol and retinol. The number of experiments is indicated in Table 3.

TABLE 4

Estimated Distribution of Neutral Lipids in Fat-storing, Endothelial, Kupffer and Parenchymal Cells Calculated on the Basis of Data Determined by TLC and HPLC[a]

	TLC					HPLC
Cell fraction	Free fatty acids	Free cholesterol	Triglycerides	Retinyl/cholesteryl esters	Total neutral lipid	Retinyl esters
Fat-storing cells	15	8	3	37	10	83
Endothelial cells	3	4	1	3	2	2
Kupffer cells	2	3	1	2	1	1
Parenchymal cells	80	85	95	58	87	14

[a]TLC, thin layer chromatography; HPLC, high performance liquid chromatography. Values represent the estimated percentage distribution of the total amount of each component in each of the four types of isolated liver cells/g liver (or in the total liver). Values were tested for significance using the unpaired t-test (two-tail).

significantly lower in parenchymal cells and even more so in Kupffer and endothelial cells (Table 3 and Fig. 3). In all these cell types retinyl esters were only a minor component (Table 1).

The best estimates for the number of each cell type present per gram of liver in 12-mo-old female BN/BiRij rats are 108×10^6 parenchymal cells, 16×10^6 fat-storing cells, 19×10^6 endothelial cells and 9×10^6 Kupffer cells (4,6). On the basis of these data, it was calculated that the recoveries of the neutral lipid classes after cell isolation varied between 44% (for triglycerides and esters of retinol and cholesterol) and 58% (for free cholesterol). The recovery of retinoids determined by HPLC was calculated to be 74%. All these calculated recoveries are in agreement with the recoveries reported on other substances using the same isolation procedure. The one exception was the recovery of free fatty acids, which was 7% only. This discrepancy is possibly caused by the metabolism of free fatty acids during the isolation procedure or the generation of free fatty acids during liver homogenization. Similar calculations showed that parenchymal cells contained 80% of the free fatty acids, 85% of the free cholesterol, 95% of the triglycerides and 58% of the esters of retinol and cholesterol present in the liver (Table 4). While Kupffer and endothelial cells contributed very little to the total neutral lipids in the liver, fat-storing cells accounted for 15% of the free fatty acids and 37% of the cholesteryl and retinyl esters. On the basis of the HPLC data, the distribution of retinoids over the different liver cell types was calculated to be the following: parenchymal cells, 14%; fat-storing cells, 83%; endothelial cells, 2%; Kupffer cells, 1% (Table 4).

Phospholipid composition of isolated cell preparations. A pilot experiment showed that parenchymal cells contained 10 times more phospholipid (160 μg/10^6 cells, n = 2) than did sinusoidal cells (13-17 μg/10^6 cells, n = 5). A similar ratio was observed for the protein contents of the two cell type populations (Table 1). The phospholipid composition of the different liver cell types was very similar, i.e., phosphatidylcholine, 48-59%; phosphatidylethanolamine, 27-33%; sphingomyelin, 9-16%; and lysophosphatidylcholine, 2-7% (Fig. 4).

The recoveries of phospholipids calculated on the basis of the cellular composition of the liver were on the same order of magnitude as the recovery of neutral lipids, namely, 38, 44, 35 and 26% for phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and lysophosphatidylcholine, respectively.

Neutral lipid composition of lipid droplets. The neutral lipid composition of both lipid droplet fractions and pellet

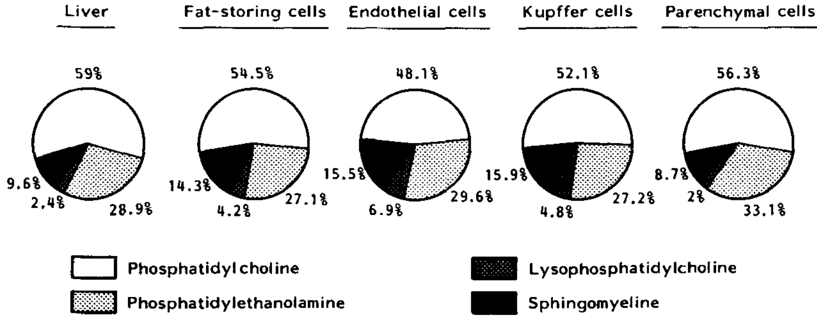

FIG. 4. Diagrams showing the phospholipid composition of fat-storing, endothelial, Kupffer and parenchymal cells and liver homogenates, expressed as percentage of the total amount of phospholipid determined. Phospholipid classes identified and included are phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and lysophosphatidylcholine.

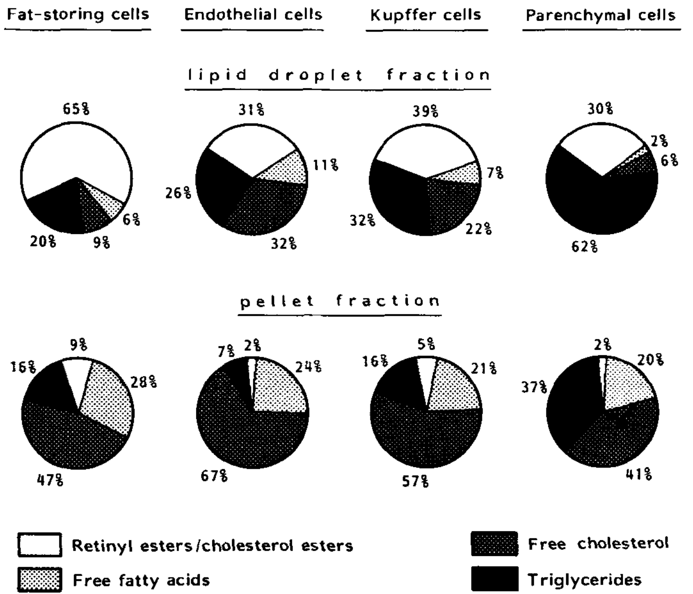

FIG. 5. Diagrams showing the neutral lipid composition of lipid droplet and pellet fractions obtained from fat-storing, endothelial, Kupffer and parenchymal cells, expressed as percentage of the total amount of neutral lipid determined. Neutral lipid classes identified and included are free fatty acids, cholesterol, triglycerides and esters of cholesterol and retinol.

fractions derived from the various purified cell types is given in Figure 5. The total neutral lipids recovered in both fractions represent 74% of the neutral lipids present in endothelial, Kupffer and parenchymal cells and 54% in fat-storing cells. The 54% recovery from fat-storing cell preparations is mainly due to a low recovery of esters (36%).

The pellet fractions of the four cell types were enriched in free fatty acids and free cholesterol but not in triglycerides and esters of retinol and cholesterol. Since the pellets consisted of the remaining cell organelles, it implies that these organelles contain mainly free fatty acids and cholesterol, while lipid droplets comprise triglycerides and esters of retinol and cholesterol (Fig. 5).

Lipid droplet fractions from both isolated endothelial cells and Kupffer cells contained hardly any lipid droplets. Most of the lipid present in these cells was recovered in their pellet fractions. Lipid droplets from parenchymal cells comprised triglycerides for 62%, contrary to fat-storing cell lipid droplets, which contained mainly retinyl and cholesteryl esters (65%). These results strengthen the data obtained in total cell fractions, showing that parenchymal cells mainly store triglycerides and some cholesteryl esters.

DISCUSSION

This report deals with the lipid composition of total liver, all major liver cell types and their lipid droplets.

The contents of free and esterified cholesterol in the serum of the rats used are in the same range as reported previously for age-matched rats (16,21). In the literature, considerable variation has been reported in serum triglyceride levels, and this relates to differences in the time of sampling and the rat strain used (16–19). Compared to these data, triglyceride levels of serum determined in

the rat strain used in this study were on the same order of magnitude.

The contents of total cholesterol, triglycerides and phospholipids in total liver homogenates were also comparable to those reported for age-matched rats (18–20). The retinyl ester content of the 12-mo-old rat livers used in this study is in the same range as values reported in the literature using modern HPLC techniques (4,22,23). Plasma retinol and retinyl ester levels were normal (24).

Lipid droplets were isolated from cell homogenates in a single centrifugation step. The sizes of isolated parenchymal cell lipid droplets were similar to those reported for isolated hepatic (25) and parenchymal cell lipid droplets (26). Isolated parenchymal cell lipid droplets showed an electron dense rim, which could be indicative of the presence of a unilamellar membrane (26). Fat-storing cell lipid droplets were more translucent. A thin electron-dense layer was often observed on the periphery, which was sometimes associated with residual cytoplasm. These droplets were larger than those observed in vivo (27,28), in cultured cells (29) and in freshly isolated fat-storing cells (30). Large droplets might have been generated by fusion of smaller droplets during the isolation procedure, since no limiting membrane was observed. Wake (5,27) reported the presence of membrane-bound (type I) and nonmembrane-bound (type II) lipid droplets in rat fat-storing cells. Type I lipid droplets correspond to lipid droplets surrounded by lysosomes (31,32). The type I lipid droplets are thought to play a role in the generation (30) or digestion (31) of fat-storing cell lipid droplets. The digestion of lipid droplets was mainly observed in hypervitaminosis A (31). Since no limiting membrane was observed to surround the lipid droplets in our studies, it seems most likely that the neutral lipid composition of these lipid droplet fractions represents the storage conditions for retinoids in normal rats.

The data presented here showed a similar distribution of the major phospholipids for all cell types tested. These data are in good agreement with those reported by Gabellec et al. (7) on the phospholipid composition of parenchymal, Kupffer and endothelial cells.

Fat-storing cells are comparable to endothelial and Kupffer cells in both size and protein content. Fat-storing cells, however, contain significantly more free cholesterol than do endothelial cells. These data indicate either that fat-storing cells also contain cholesterol as a lipid droplet constituent or that these cells contain considerably more cell membranes. A significant but small portion of this free cholesterol is indeed still present in the lipid droplet fractions obtained from these cells (8). Electron microscopic observations in humans (33) and rats (34) show that fat-storing cells possess numerous large extensions that allow extensive contacts with all liver cell types and the space of Disse. Blouin et al. (1) showed that both fat-storing cells and endothelial cells indeed contained (as a percentage) more plasma membranes than did Kupffer cells.

The results presented in this paper provide additional data on some aspects of the functions of different cell types in the liver. As shown in this and previous papers (4,6), fat-storing cells are the main retinoid storage sites in the liver. The two cell types most important in handling the vitamin are parenchymal and fat-storing cells (4,6). Parenchymal cells take up chylomicron remnant particles, which transport dietary retinoids mainly as retinyl esters (35). The parenchymal cells are also able to hydrolyze these esters by retinyl palmitate hydrolase activity and to transport retinol within the cell by the cellular retinol binding protein (6,36). Moreover, parenchymal cells synthesize and secrete the retinol binding protein that transports retinol in the blood (37,38). Fat-storing cells store the vitamin as retinyl esters and are also able to hydrolyze retinol from these stores by retinyl palmitate hydrolase activity and to transport retinol within the cell by the cellular retinol binding protein (6). Blomhoff et al. (36) showed that the fat-storing cells and parenchymal cells of rat liver also contain the acyl CoA:retinol acyltransferase activity, which is necessary to esterify retinol with a free fatty acid. These data indicate that both cell types are able to generate and utilize free fatty acids for handling the vitamin. It is shown in this paper that the majority of the neutral lipids present in fat-storing cells consists of retinyl esters (8), but substantial amounts of triglycerides are also present. It is tempting to speculate that these triglycerides serve as a storage site for the free fatty acids needed for the esterification of retinol.

The majority of neutral lipids in parenchymal cells consisted of triglycerides; this is related to the liver's important function in formation and secretion of very low density lipoproteins (39–41). Cholesteryl esters are also present in the parenchymal cell lipid droplet fractions as a consequence of the parenchymal cell's role in cholesterol synthesis and lipoprotein uptake.

Lipid droplet fractions isolated from endothelial and Kupffer cells contained minor amounts of neutral lipids. Most neutral lipids were recovered in the pellet fraction mainly as cholesterol and free fatty acids. Sinusoidal endothelial and Kupffer cells were reported to play a significant role in the uptake and metabolism of several lipoproteins (42,43). Lipids probably are not stored but degraded by means of lysosomal enzymes present in these cells (43–45).

Data presented in this study show that the cell types present in liver differ considerably in their neutral lipid composition. These differences are more apparent in the specific lipid storage organelle, the lipid droplet. It is concluded that the differences in neutral lipid composition described in this paper correlate well with the functions of the various cell types in liver lipid metabolism.

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METHODS

Two Procedures to Remove Polar Contaminants from a Crude Brain Lipid Extract by Using Prepacked Reversed-phase Columns

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Two procedures were developed using prepacked, reversed-phase columns (Bond Elut) for the separation of lipids from water-soluble contaminants. A crude lipid extract from brain tissue was diluted stepwise with a methanol/water (method 1) or a methanol/saline (method 2) mixture and, with each step, was passed through the column. As the polarity of the solvent was increased, all lipids became bound to the column, while the water-soluble compounds remained in the eluate. After three subsequent dilutions and column elutions, the eluate containing the more polar contaminants was discarded. The bound lipids were then eluted with a small volume of chloroform/methanol (1:2, v/v). Alternatively two fractions were eluted, the first fraction eluted with methanol/water (12:1, v/v), contained gangliosides, phosphatidylserine, phosphatidylinositol, phosphatidic acid and sulfatides. The second fraction, eluted with chloroform/methanol (1:2, v/v), contained all remaining phospholipids, cerebrosides and cholesterol. For both methods a quantitative recovery of cholesterol and phospholipids was obtained. In method 2, when water was replaced by saline in the dilution solvent mixture, gangliosides were also bound and quantitatively recovered.
Lipids 22, 274–277 (1987).

For the extraction of lipids from various tissues, chloroform/methanol solvent mixtures are most commonly used (1,2). The crude extracts obtained contain small amounts of nonlipid substances that can interfere with the subsequent quantitative determination of the various lipids. Removal of the nonlipid contaminants is often done either by partitioning against a salt solution (1) or by using chromatography on a dextran gel column (3). These methods become quite elaborate when numerous samples are to be run with several solvent evaporations.

Recently, reversed-phase columns have been shown useful for isolation and purification of gangliosides from the upper phase obtained after partitioning (4). They have also been used for separation of radioactive lipid precursors from product complex lipids in a microsomal preparation, where the entire reaction mixture was poured onto the column without a previous extraction of the lipids (5). In both methods, the solvent composition was that of a theoretical upper phase (TUP) (chloroform/methanol/aqueous salt solution, 3:48:47, v/v/v). Such a polar solvent dissolves gangliosides readily, but not other lipids.

The present report starts with a standard chloroform/methanol total lipid extract. Two methods were developed according to the same general principle. By a stepwise dilution of the crude extract with a methanol/water (method 1) or methanol/saline (method 2) mixture followed by elution through a C-18 reversed-phase column, the complete lipid sample can be bound to the column while polar contaminants remain in the eluate. The lipid sample can then be easily eluted with a less polar solvent. The simplified procedure (method 1) results in a purified lipid sample, similar to the lower phase of Folch et al. (1). In the second method (method 2), where water was substituted with saline (4), gangliosides also were completely bound to the column. Two fractions were eluted in method 2, similar to those obtained after ion-exchange chromatography (13).

MATERIALS AND METHODS

Chemicals. All chemicals and solvents were of reagent grade. C-18 Bond Elut columns (2.8 ml, 500 mg sorbent) were from Analytichem International (Harbor City, California) and 0.25-mm thin layer chromatography (TLC) plates, Silica Gel 60, were from Merck (Darmstadt, Federal Republic of Germany). [^{3}H]Glucose and [^{14}C]-dipalmitoyl phosphatidylcholine were purchased from Amersham (Buckinghamshire, England). The elution apparatus was similar to that previously described (6). Standard luer-lock stopcocks were attached to the lid of the apparatus.

Tissue preparation. Lipids were extracted from the tissue with chloroform/methanol. Approximately 2 g frozen brain was homogenized in 20 ml methanol, to which 10 ml chloroform was added. The homogenate was centrifuged at 1000 × g for 10 min, and the pellet was then reextracted by the same procedure. All steps were carried out at 0–4 C.

Method 1. To 6 ml of the crude extract, corresponding to about 200 mg fresh brain, 8 ml of methanol/water (1:1, v/v) was added and mixed to homogeneity. If the lipid content of the samples was high, the mixture became slightly turbid at the first dilution. This did not affect the recovery. The whole mixture was poured onto a C-18 Bond Elut column equipped with a 50-ml reservoir. The column was preequilibrated with 2 ml methanol. New columns were washed with 6 ml chloroform/methanol (1:2, v/v) before use. Column elution was carried out under a constant gentle vacuum with an elution speed of 1–2 ml/min. Another 8 ml of methanol/water (1:1, v/v) was added to the eluate, mixed and passed through the same column. The resulting eluate was once again mixed with 8 ml of methanol/water and passed through the column as before. As soon as all liquid had passed the columns, the stopcocks were closed, to keep elution volumes reasonably constant and to avoid changing the column properties by allowing the columns to dry. The last eluate was normally discarded before elution of the bound lipids. A 5-ml container was mounted on top of the column, and the lipids were eluted at a moderate rate with two portions of 4.0 ml chloroform/methanol (1:2, v/v). This final eluate was used for quantitative analyses of lipid classes. The final elution from the column was also done with 4 × 2-ml

portions to verify the completeness of the elution. The columns could be reused 3-5 times without any appreciable decrease in column flow. The elution speed was drastically decreased if the columns were overloaded; thus sample breakthrough hardly occurred.

Bidirectional qualitative TLC was done on the purified extract (9). In the first direction, chloroform/methanol/water was used in the proportions 65:25:4 and in the second direction chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1, v/v/v/v/v) was used. The plates were exposed to hydrochloric acid vapors for 2 min between the first and second directions. Lipid spots were visualized by charring with a cupric sulfate reagent (10). Identification of the various lipid spots was done by comparing with appropriate standard mixtures and by their characteristic fatty acid patterns upon gas-chromatographic analysis. In addition, ninhydrin spray reagent (Merck) was used to detect nonlipid material.

A reference method was compared to method 1. From the same crude extract as used for method 1, 6-ml samples were purified by partitioning in principle according to Folch et al. (1). These samples were first taken to dryness by rotary evaporation and then redissolved in chloroform/methanol (2:1, v/v). To the resulting extracts, 0.2 volumes of 0.9% NaCl in water were added, and the tubes were vortexed and centrifuged at 2000 × g for 10 min. The upper phase was removed by aspiration, and the lower phase was taken to dryness and then redissolved in 8 ml chloroform/methanol (1:2, v/v).

Cholesterol was determined on the extracts from method 1 and from the reference methods according to Franley and Amador (7). Their phosphorus contents were also determined (8).

An experiment to study the recovery of a labeled lipid and a water-soluble residue was carried out by adding [^{3}H]labeled glucose (50 μCi) and [^{14}C]labeled dipalmitoyl phosphatidylcholine (5 μCi) to a 60-ml sample of crude brain-lipid extract. Six-ml aliquots of the extract were diluted and passed through the Bond Elut columns according to the procedure described above. Radioactivity was determined in both the crude lipid extract and in the lipid fraction eluted from the column using double label liquid scintillation counting.

Method 2. Method 2 was a slight modification of method 1. After a first dilution of the crude extract with methanol/water (1:1, v/v) and a column elution step identical to method 1, the second and third dilutions and column elution steps were carried out with methanol/0.9% saline (1:1, v/v). The column was then washed with 2 ml water to remove salt before elution of the lipids. The lipids were then eluted in two fractions, the first (I) with two 4-ml portions of methanol/water (12:1, v/v) and the second (II) with two 4-ml portions of chloroform/methanol (1:2, v/v).

The reference partitioning method was performed as described above for method 1, except that after aspiration of the upper phase the surface was rinsed with two 1-ml portions of TUP. The combined upper phases were diluted with methanol to 8 ml.

Unidirectional TLC was done on all fractions in method 2 and reference methods. The plate was developed in chloroform/methanol/water (65:25:4, v/v/v) and visualized by charring (10). The ganglioside-containing fractions were also developed in chloroform/methanol/0.25% $CaCl_2$ (60:35:8, v/v/v). Identification of the individual lipid bands was done as for method 1. To separate phosphatidylserine and phosphatidylinositol, the same bidirectional TLC was performed as described for method 1. Gangliosides were identified by development of blue color after charring with a modified reagent, which also contained 1 ml 3% resorcinol solution added to 100 ml of the original reagent (10) used in method 1. Individual gangliosides were identified tentatively.

Phospholipids and cholesterol were determined on fractions I and II of method 2 and on the lower phase of the reference method as described above. Sialic acid was analyzed, without a prior dialysis, by the method of Svennerholm (11), modified by Miettinen and Takki-Luukkainen (12). A slight modification was made in adding 1-butanol to the samples prior to color development. Thus, samples that also contained lipids other than gangliosides were completely dissolved in the reaction mixture. Aliquots of the lipid extracts were sampled into narrow 10-ml screw cap tubes and evaporated to dryness in a stream of nitrogen. To the dried samples, 0.38 ml 1-butanol was added, and the tubes were vortexed. Then 1.2 ml water or sialic acid standard solution in water, containing 7.5, 15.0 and 22.5 μg sialic acid, followed by 1.2 ml resorcinol reagent was added, and the tubes were vortexed again. The butanol was completely dissolved in the reaction mixture. The tubes were heated in a boiling water bath for 30 min. To the tubes, when they had cooled, 2.13 ml butyl acetate was added. The tubes were vortexed and then centrifuged at 2000 × g for 5 min. The clear upper layer was read on a spectrophotometer at 580 nm. Since a slight discoloring was observed in the crude extract samples, sample blanks of the corresponding amount of ganglioside-free brain lipids were run throughout the procedure. The sample blank readings were subtracted from the sample readings.

RESULTS

The simplified reversed-phase column procedure (method 1) presented in this report and the reference partitioning method resulted in almost the same quantitative recovery of cholesterol and phospholipids. (Total phospholipids [mg ± S.D.]: method 1, 12.4 ± 0.28; reference method, 12.0 ± 0.13. Cholesterol: method 1, 4.83 ± 0.14; reference method, 4.76 ± 0.06.) The recovery of radioactive phosphatidylcholine was high and the added [^{3}H]labeled glucose was effectively washed away from the column (Table 1). Almost all the bound lipid (>99%) was eluted

TABLE 1

Recovery of [^{14}C]Phosphatidylcholine and [^{3}H]Glucose from Aliquots of a Crude Brain Lipid Extract Purified with 3-ml Bond Elut Columns (Method 1)

	[^{14}C]dpm ± S.D. (25-μl sample)	%	[^{3}H]dpm ± S.D. (25-μl sample)	%
Crude extract (n = 2)	3400 ± 71.6	100	34035 ± 467	100
Method 1 (n = 4)	3514 ± 47.3	103	−189 ± 13.1[a]	−0.6

[a]Negative value because of a slight overcompensation of spillover from the high energy isotope.

TABLE 2

Lipid Content of a Rat Brain Lipid Aliquot Purified with 3-ml C-18 Bond Elut Columns (Method 2) or by Partitioning

	Total phospholipids (mg ± S.D.)	Cholesterol (mg ± S.D.)	Sialic acid (μg ± S.D.)
Reference method (n = 4)			
Lower phase	13.6 ± 0.11	5.78 ± 0.19	14.2 ± 5.77
Upper phase	—	—	170.4 ± 16.43
Method 2 (n = 4)			
Fraction I	3.2 ± 0.06	0.18 ± 0.03	197.1 ± 1.95
Fraction II	10.5 ± 0.44	5.56 ± 0.09	0.0 ± 2.41
Crude extract			207.4

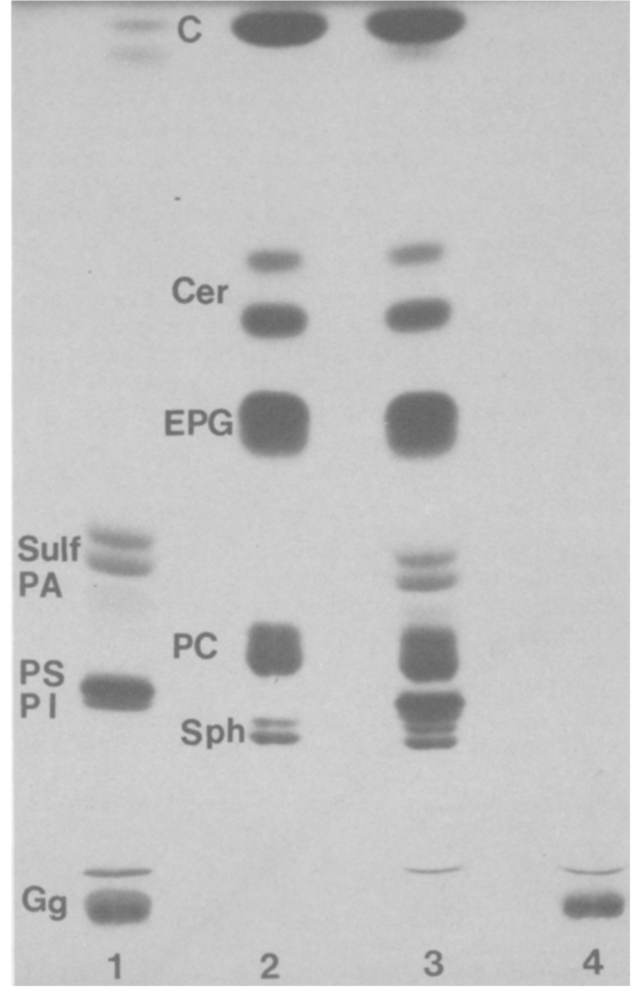

FIG. 1. A unidirectional thin layer chromatograph of a brain lipid extract purified with method 2 on C-18 Bond Elut reversed-phase columns and of a reference method. The plate was developed in chloroform/methanol/water (65:25:4, v/v/v) and visualized by charring. Lane 1, fraction I; lane 2, fraction II; lane 3, lower phase of reference method; lane 4, upper phase of reference method; C, cholesterol and neutral lipids; Cer, cerebrosides; Sulf, sulfatides; EPG, ethanolamine phosphoglycerides; PC, choline phosphoglycerides; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin; Gg, gangliosides.

with the first two portions of chloroform/methanol (1:2, v/v), and the final portion eluted contained no detectable lipid. However, the eluate of method 1 was more contaminated by gangliosides than the lower phase of the partitioning method (about 20% of the total sialic acid content). Ninhydrin positive spots were not observed after TLC other than for serine and ethanolamine phosphoglycerides (data not shown).

Besides a high recovery of cholesterol and phospholipids in method 2, a high recovery and a low variation between samples was obtained for the gangliosides in this method (Table 2). As indicated in Figures 1 and 2, two well-characterized fractions were eluted in method 2. Fraction I contained gangliosides, acidic phospholipids (phosphatidylserine, phosphatidylinositol, phosphatidic acid) and sulfatides, and fraction II neutral phospholipids (ethanolamine and choline phospholipids, sphingomyelin and cerebrosides). In fraction II, 97% of the cholesterol was eluted; 3% of the cholesterol or cholesterol derivatives was eluted with fraction I. No contamination of gangliosides could be observed in fraction II (Table 2 and Fig. 2). However, in the reference method there was a lower recovery of the bands corresponding to G_{M1} and G_{M3} gangliosides compared to method 2 (Figs. 1 and 2).

DISCUSSION

Using the common partitioning method according to Folch et al. (1), the lipids and the contaminants exist in two liquid phases of about equal volume. Therefore, after aspiration of the upper layer, the surface of the lower layer has to be washed repeatedly to ensure dilution of residual contaminants. In so doing, there is always the possibility of removing some of the lower phase or interfacial fluff. However, with the reversed-phase column methods, the lipids become bound to the column and occupy a very

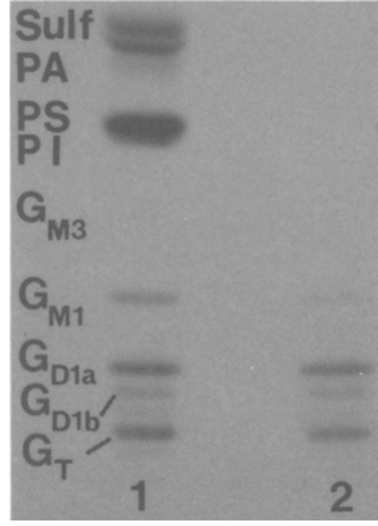

FIG. 2. A unidirectional thin layer chromatograph of a brain lipid extract purified with method 2 on C-18 Bond Elut reversed-phase columns and of a reference method. The plate was developed in chloroform/methanol/0.25% $CaCl_2$ (60:35:8, v/v/v) and visualized by charring. Lane 1, fraction I; lane 2, upper phase of reference method; Sulf, sulfatides; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; gangliosides G_{M3}, G_{M1}, G_{D1a}, G_{D1b} and G_T (nomenclature by Svennerholm).

small volume. The polar contaminants remain in solution in the very diluted aqueous phase. The bound lipids can then be directly eluted with a known volume of a less polar solvent and quantitatively determined.

The principle for the presented methods was to start with a lipid extract in a less polar solvent and, by stepwise dilution, to obtain a successively more polar solvent without phase separation. However, phase separation will occur if the water content or the salt concentration is further increased. As a consequence, the first dilution of the extract cannot be done with saline or with a higher water content.

A successful purification of the lipid extract by the reversed-phase column methods was evident from the complete removal of added labeled glucose and the absence of ninhydrin positive spots other than lipids after TLC. The presented methods were also as accurate as the reference method for quantitative lipid determinations. For gangliosides, method 2 had an even better recovery than the reference method. An advantage of the presented methods was that they take less time, since several samples can be run in parallel and no solvent evaporations are necessary (max. 90 min from crude to purified and/or fractionated extract). Furthermore, there is an obvious advantage in the direct separation of the components in the two fractions I and II. Thus a separate ion-exchange step to separate acidic from neutral phospholipids (13) is unnecessary. We feel that the methods presented here for the purification and partial fractionation of crude lipid extracts have several applications for routine lipid analytical work and that they are very suitable for automation.

ACKNOWLEDGMENTS

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COMMUNICATIONS

Fate of 7,12-Dimethylbenz(*a*)anthracene Absorbed from the Rat Intestine and Transported in Chylomicrons

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Chylomicrons obtained from the thoracic duct of rats fed [^{3}H]7,12-dimethylbenz(*a*)anthracene (DMBA), a polycyclic aromatic hydrocarbon, were infused intravenously into rats with bile fistulas. Over 17 hr, 55.9 ± 3.2% (mean ± SEM) of the radioactivity was recovered in bile and 6.7 ± 0.5% in urine. Minor amounts were deposited in liver, kidneys and epididymal fat pads. Injection of DMBA in ethanolic solution gave a similar pattern, while biliary DMBA metabolites resulted in higher recovery in urine and lower recovery in fat. In conclusion, the major part of chylomicron DMBA is rapidly excreted via the biliary route, while a fraction is probably retained in adipose tissue.
Lipids 22, 278–281 (1987).

Polycyclic aromatic hydrocarbons (PAH), which constitute a major class of lipophilic xenobiotics of environmental and toxicological importance, are formed during incomplete combustion of fossil fuels and as a result of food processing (1). This group of nonpolar compounds contains several well-recognized carcinogens, such as benzo(*a*)pyrene, 3-methylcholanthrene and 7,12-dimethylbenz(*a*)anthracene (DMBA). DMBA is a potent mammary carcinogen, extensively used in experimental studies in rodents (2). It is readily absorbed from the gastrointestinal tract when presented as a solute in dietary lipid (3). The major proportion of DMBA taken up by the enterocyte is metabolized and transported to the liver via the portal venous blood (4), which is followed by biliary excretion and enterohepatic circulation of the metabolites. However, some of the absorbed DMBA passes through the enterocyte unmetabolized and is transported by the lymph as a solute of the triglyceride core of chylomicrons (5). The fate of DMBA that escapes metabolism in the enterocyte is of interest, since the compound has the opportunity to reach peripheral tissues. The aim of this investigation was to examine the fate of DMBA reaching the systemic circulation as a chylomicron solute.

MATERIALS AND METHODS

Chemicals. [G-^{3}H]7,12-dimethylbenz(*a*)anthracene (Amersham, Little Chalfont, United Kingdom) was 97.8% pure according to the manufacturer. Unlabeled DMBA was obtained from Sigma (St. Louis, Missouri).

Preparation of chylomicrons containing [^{3}H]DMBA from "donor" rats. Male Sprague-Dawley rats (ALAB, Stockholm, Sweden) weighing 250–350 g and fed a standard pellet diet were used for the study. On the day before surgery, solid food was withdrawn, and rats were allowed to drink ad libitum a glucose-saline solution containing 2.5% glucose, 0.5% NaCl and 0.05% KCl (w/v). Under ether anesthesia, the infradiaphragmatic portion of the thoracic duct was cannulated with clear vinyl tubing (SV 31, id 0.50 mm, od 0.80 mm; Dural Plastics and Engineering, Dural, N.S.W., Australia). A duodenal catheter of the same tubing was introduced through the antrum of the stomach. It was secured with a purse-string suture in the stomach wall, and the tip was located about 2 cm distal to the pyloric sphincter. Both catheters were led out through stab wounds in the flank. Postoperatively, the animals were kept in restraining cages at 30 C and infused intraduodenally with the glucose-saline solution at 1.8 ml/hr. Approximately 24 hr after surgery, the rats received over one hour an intraduodenal infusion of 1 ml corn oil containing 5 nmol [^{3}H]DMBA (sp act 50 μCi/nmol). Lymph was collected in 30-min samples, and the samples containing the highest amounts of radioactivity (about 2–4 hr after start of the [^{3}H]DMBA-corn oil infusion) were pooled. The lymph was diluted with a solution containing 1.1% NaCl and was centrifuged in a 6 × 14 ml swing-out rotor for 2 hr at 10 C in polycarbonate tubes at 25,000 rpm (g_{av} = 75,000) in an MSE prepspin 50 centrifuge. The supernatant layer containing the chylomicrons was harvested after tube slicing. Thin layer chromatography (TLC) of chylomicrons was carried out on silicic acid plates (Kieselgel 60 on alumina; Merck, Darmstadt, Federal Republic of Germany) to determine the identity of the ^{3}H activity. Aliquots of the chylomicron fraction were put directly on the plates, which were developed with ether/hexane (5:95, v/v). Unlabeled DMBA used as reference was visualized with iodine vapor, and the corresponding area containing [^{3}H]DMBA was cut out and radioactivity was measured after addition of 10 ml of Emulsifier Scintillator 299 (Packard Instruments Co., Downers Grove, Illinois).

Intravenous infusion of chylomicrons containing [^{3}H]DMBA into "recipient" rats. Recipient rats were treated in the same way as donor rats before surgery. Under ether anesthesia, the bile duct was cannulated near the hilum of the liver with a 5-mm-long polyethylene tubing (PP 10; 0.28 mm id, 0.61 mm od; Portex, Hithe, United Kingdom) connected to an SV 31 clear vinyl tubing, which was led out through a stab wound in the flank. An intravenous infusion was set up through a tail vein by passing a PP 10 catheter through an 18-gauge hypodermic needle (Yale no. 5196; Becton Dickinson, Mississauga, Ontario, Canada) and securing the catheter with a drop of cyanoacrylate glue. After surgery, the rats were kept in restraining cages at 30 C, and during the whole experiment (except when radiolabeled infusates were administered) were given an intravenous infusion of 145 mM NaCl and 5 mM KCl at 0.6 ml/hr. The day after surgery the recipient rats received a tail vein infusion at a rate of 0.6 ml/hr over 1.5 hr of radiolabeled chylomicrons (containing ca. 10 pmol DMBA; chylomicrons

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were prepared from lymph of 1–3 donor rats) resuspended in a solution containing 145 mM NaCl and 5 mM KCl (this infusate is denoted as DMBA-CM in the following). Bile samples were continuously collected before, during and after this infusion. Urine was also continuously collected from the start of the chylomicron infusion. For comparison, a group of rats with biliary fistulas and intravenous tail vein catheters received over 1.5 hr an infusion of rat serum to which had been added an ethanolic solution of [^{3}H]DMBA (ca. 5 nmol [^{3}H]DMBA in 50 μl ethanol + rat serum to a total volume of 1 ml [DMBA-ES infusate]). Bile, urine and tissue samples were collected as described above. An additional group of rats received over 1.5 hr an intravenous infusion of bile (DMBA-BM infusate; containing ca. 200 pmol of DMBA metabolites) collected from the DMBA-ES rats. Consequently this bile (collected during the first 7 hr after start of the DMBA-ES infusion) contained biliary metabolites of [^{3}H]DMBA. After 22 hr, the DMBA-ES and DMBA-BM rats were killed. The liver, kidneys and epididymal fat pads were excised and weighed, and samples were taken for radioactivity counting. For practical reasons, due to the time needed for preparation of fresh chylomicrons, the DMBA-CM rats were killed after 17 hr instead of 22 hr.

Radioactivity measurements. Aliquots of lymph, bile and urine were brought to a total volume of 1 ml by addition of distilled water and were mixed with 10 ml Emulsifier Scintillator 299 (Packard) for liquid scintillation counting in a Packard Tri-Carb 4530. Aliquots of tissue (ca. 100 mg) were dissolved at 55 C overnight in 1 ml of Soluene 350 (Packard) before addition of 10 ml Dimilume-30 (Packard).

Statistics. Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's t-test.

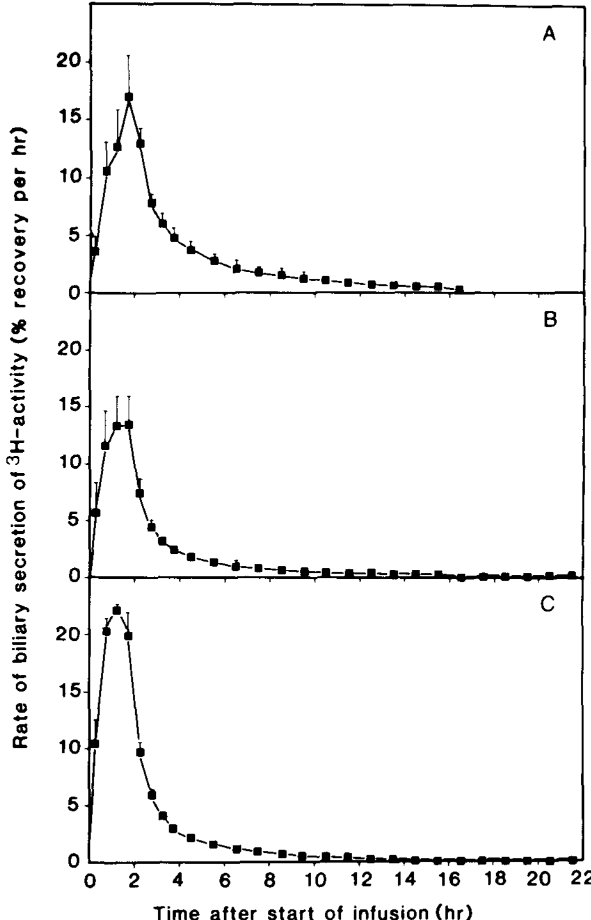

FIG. 1. Rate of biliary secretion of ^{3}H activity as a function of time after start of a 1.5-hr intravenous infusion of [^{3}H]DMBA-labeled chylomicrons (A), of [^{3}H]DMBA in ethanol solution mixed with rat serum (B) or of biliary [^{3}H]DMBA metabolites in bile (C) to rats with bile fistulas. Secretion rate is expressed as % recovery/hr of the total dose infused. Points represent mean ± SEM of values obtained from 6 (A and C) or 7 (B) rats.

RESULTS

TLC showed that practically all the radiolabel (>99%) was present as the native compound in the chylomicrons obtained from rats with lymph fistulas that were fed [^{3}H]DMBA.

When DMBA-CM (chylomicrons containing DMBA) were infused intravenously, the radiolabel rapidly appeared in bile, as is shown in Figure 1A. The major part of the total amount recovered in bile was secreted within 4 hr after start of the infusion. During the following period of the experiment, the biliary secretion rate was constantly low.

A similar pattern of biliary radiolabel secretion was obtained when DMBA-ES (DMBA dispersed in ethanol and serum) (Fig. 1B) and DMBA-BM (biliary metabolites of DMBA) were infused (Fig. 1C). Figure 2 shows the cumulative recovery of radiolabel during the first 3 hr, which was higher for DMBA-BM than for DMBA-CM 3 hr after the start of infusion ($p < 0.05$). The difference in recovery after 3 hr between DMBA-CM and DMBA-ES was not significant.

Table 1 shows the recovery of ^{3}H activity in bile, urine and some tissues after intravenous infusion of DMBA-CM, DMBA-ES or DMBA-BM. In the case of DMBA-CM, the major amount was recovered in the bile (55.9 ± 3.2%), whereas a minor portion was found in the urine (6.7 ± 0.5%). Similar results were obtained when DMBA-ES was infused, whereas the urinary recovery of radiolabel in rats receiving DMBA-BM was considerably greater than in animals receiving DMBA-CM. However, the major excretory route was bile in all three groups.

DISCUSSION

The present study shows that a proportion of DMBA absorbed from the small intestine is transported unmetabolized as a chylomicron solute. The major proportion of DMBA injected intravenously as chylomicron solutes in this study was recovered in bile, and the pattern of recovery was close to that obtained when the compound was given by intravenous injection as an ethanolic solution in serum. The necessity of metabolic transformation of DMBA by the liver prior to biliary excretion could account for the fact that ^{3}H activity appeared at a slower rate after infusion of DMBA-CM or DMBA-ES compared to DMBA-BM. Relatively little radioactivity appeared in the urine when DMBA-CM or DMBA-ES was infused; this is presumably derived from polar metabolites of DMBA that have entered the systemic circulation. Tissue

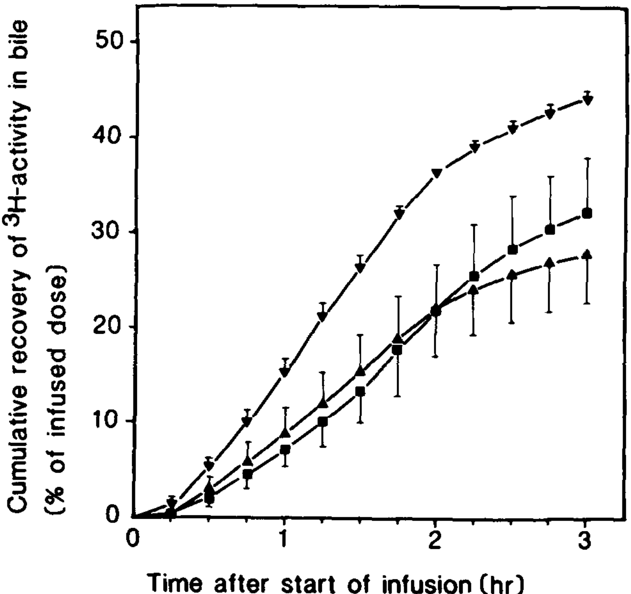

FIG. 2. Cumulative recovery of ^{3}H activity in bile as a function of time after start of a 1.5-hr intravenous infusion of [^{3}H]DMBA-labeled chylomicrons (■), of [^{3}H]DMBA in ethanol solution mixed with rat serum (▲), or of biliary [^{3}H]DMBA metabolites in bile (▼) to rats with bile fistulas. Points represent mean ± SEM of values obtained from 6 (■ and ▼) or 7 (▲) rats.

TABLE 1

Recovery of ^{3}H Activity 17 Hr (DMBA-CM) or 22 Hr (DMBA-ES and DMBA-BM) After Start of a 1.5-Hr Intravenous Infusion of [^{3}H]DMBA or Biliary [^{3}H]DMBA Metabolites to Rats with Bile Fistulas[a]

	DMBA-CM (n = 6)	DMBA-ES (n = 7)	DMBA-BM (n = 6)
Bile	55.86 ± 3.21	40.85 ± 4.85	58.40 ± 1.03
Urine	6.73 ± 0.52	3.42 ± 0.59	22.87 ± 1.06
Liver	3.73 ± 0.45	1.04 ± 0.04	2.39 ± 0.05
Kidneys	1.04 ± 0.17	0.20 ± 0.04	2.94 ± 0.11
Epididymal fat pads	0.55 ± 0.05	0.13 ± 0.04	0.03 ± 0.01
Total recovery	67.91 ± 4.03	45.64 ± 4.94	86.63 ± 1.85

[a]Data are expressed as mean ± SEM. DMBA-CM, [^{3}H]DMBA-labeled chylomicrons obtained from thoracic duct lymph from rats given an intraduodenal infusion of [^{3}H]DMBA as a solute in corn oil; DMBA-ES, [^{3}H]DMBA in ethanol solution mixed with rat serum before the intravenous infusion; DMBA-BM, biliary metabolites of [^{3}H]DMBA obtained from rats with bile fistulas that were given an intravenous infusion of [^{3}H]DMBA in ethanol solution mixed with rat serum.

levels at 17 hr were low; there was, however, a small deposition of DMBA in epididymal adipose tissue, which was greater than that observed when the compound was given in ethanolic solution. Only trace amounts of radiolabel were recovered in fat tissue when the biliary metabolites of DMBA were given. From Figure 1 it is evident that after ca. 12 hr the difference in biliary secretion rate of ^{3}H activity between the three experimental groups is negligible. Consequently, the fact that the rats were killed after 17 or 22 hr does not affect the results of the experiment, since no significant changes in tissue distribution occur between 17 and 22 hr.

There is a substantial proportion of administered radiolabel that was not accounted for. This was presumably distributed among other tissues. With the assumption that DMBA is handled similarly by all forms of adipose tissue, which constitutes 7.08% of body weight (6), the total recovery of ^{3}H activity in adipose tissue has been estimated to be 15.7 ± 4.4% 17 hr after infusion of DMBA-CM. One tissue not examined that might have contained appreciable amounts of the compound is lung, since a study of benzo(*a*)pyrene disposition after intravenous injection of chylomicrons mixed with the radiolabeled hydrocarbon showed that lung contained higher amounts of radioactivity per unit weight of tissue than liver, kidneys, brain, muscle, fat or ovaries (7). Another possibility not examined was fecal excretion as a result of transintestinal secretion.

Many nutrient and non-nutrient lipid-soluble compounds in the diet travel in lymph as core solutes in chylomicrons. These include fat-soluble vitamins, cholesteryl esters, DDT (8) and PAH (9). The major part of chylomicron triglyceride is taken up in extrahepatic tissue as a result of lipoprotein lipase activity; >80% of the cholesteryl esters of chylomicrons are taken up by the liver via chylomicron remnants (10). Vitamin A esters are a relatively nonexchangeable component of chylomicrons and their remnants and are taken up quantitatively by the liver with the remnants (11). By contrast, significant amounts of vitamin D_3 are transferred from chylomicrons to other plasma fractions (12). Whether DMBA remains with chylomicron remnants like vitamin A esters or exchanges with other plasma lipoprotein fractions is not known. Benzo(*a*)pyrene has been reported to transfer from chylomicrons to the low density lipoprotein (LDL) and the high density lipoprotein (HDL) fractions (7,13).

The present results suggest that DMBA in chylomicron triglyceride is largely available for liver metabolism followed by biliary excretion, but that deposition of the compound in adipose and other tissues also has to be considered. However, several aspects of transfer of DMBA between lipoprotein classes as well as uptake into and elimination from different organs remain to be investigated before the principles determining the fate of this PAH can be established.

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Effect of Environmental Temperature Changes on Liver Δ6 and Δ5 Fatty Acid Desaturases Depressed by Hyperglycidic Diet on Male Rats

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Male rats maintained on a control diet at 24 C and shifted to 12 C for five days showed no modification in Δ6 or Δ5 desaturase activity. When Δ6 and Δ5 desaturase activities were diminished by a hyperglycidic diet, shifting to cold increased both enzymatic activities. In general, modifications observed in liver microsomal fatty acid composition are consistent with those associated with the enzymatic desaturation activity.
Lipids 22, 282-284 (1987).

A response of poikilotherms to environmental temperature changes is the alteration of membrane lipid composition associated with the degree of unsaturation of fatty acids (1,2).

While the metabolic basis for increasing unsaturation is not entirely clear, there is evidence that cold-adapted organisms show higher rates of production of unsaturated acids than do warm-adapted organisms (3-5). Thus, enhanced rates of desaturation may account for some of the fatty acid composition changes that accompany cold adaptation. In eucaryotic organisms, the endoplasmic reticulum could constitute a kind of self-regulated system for maintaining an optimal physical state by means of activation or deactivation of desaturation reactions.

The effect of temperature on membrane fluidity is not as obvious in homeothermic animals as in poikilotherms. In previous studies done in this laboratory (6) in which female rats were adapted to warm temperature (30-32 C) for 20-25 days and then shifted to colder temperature (13-15 C) for different periods of time, Δ6 and Δ5 desaturase activities increased 24 hr after cold exposure. Male rats adapted under the same conditions did not show significant changes in Δ6 desaturase activity.

Since a hyperglycidic diet (HD) depressed Δ6 desaturase activity, it was thought that, starting from decreased enzymatic activity levels, it would be possible to observe in male rats an increase of this enzyme due to cold temperature.

For this purpose, male rats were fed on HD and shifted to different temperatures. Activities of Δ6 and Δ5 desaturase and hepatic microsomal fatty acid composition were determined.

MATERIALS AND METHODS

[1-^{14}C]Linoleic acid (54.7 mCi/mmol, 98.5% radiochemically pure, 1% *trans* isomer) and [1-^{14}C]eicosa-8,11,14-trienoic acid (54.9 mCi/mmol, 99% radiochemically pure, <3% *trans* isomer) were purchased from New England Nuclear (Boston, Massachusetts). Cofactors used for the enzymatic reactions were provided by Sigma Chemical Co. (St. Louis, Missouri) and all unlabeled fatty acids were from Nu-Chek Prep (Elysian, Minnesota). All chemicals and solvents were of analytical grade.

Animals and their treatment. White male Wistar rats, 60-75 days old and weighing 190-220 g, were maintained on Cargill rat chow and housed individually in temperature-controlled rooms at 24 ± 1 C. All animals were subjected daily to 12 hr light and 12 hr darkness, with midnight being the midpoint of the dark period.

The animals were divided into two groups of 15 animals, fed different isocaloric diets (4.4 cal/g food) and maintained for two days at 24 ± 1 C. Food and tap water were given ad libitum. One group received a control diet (CD) and the other HD. After this period, each group was subdivided into three groups of five animals, each with the same dietary conditions. One group from each diet was shifted to a cold room at 12 ± 1 C (group I [CD] and group II [HD]); two other groups were maintained at the same temperature (group III [CD] and group IV [HD]) and the last two groups transferred to a warmed room at 31 ± 1 C (group V [CD] and group VI [HD]). After five days under these conditions, the animals were killed at 8 a.m. to avoid circadian effects (7), and liver microsomes were isolated as described previously (6).

The diets contained equal amounts of salts and vitamins (8), casein, maize oil, glucose and starch, which were used as sources of protein, fat and carbohydrate, respectively. In the CD, 55, 20 and 25% of the calories were supplied as carbohydrate (100% starch), protein and fat. The distribution of calories in the HD was as follows: 69% carbohydrate (62% starch and 38% glucose), 6% protein and 25% fat.

Enzymatic assays. Reactions were initiated by the addition of microsomal protein to preincubated flasks containing 0.25 M sucrose, 0.15 M KCl, 0.04 M potassium phosphate buffer (pH 7.2), 1.41 mM N-acetyl cysteine, 0.04 M KF, 1.3 mM ATP, 0.06 mM CoA, 0.87 mM NADH, 5 mM $MgCl_2$, 5 nmol [1-^{14}C]labeled acid and 45 nmol unlabeled acid in a final volume of 1.6 ml. For Δ6 desaturase assays, linoleic acid was used as substrate and 3 mg of protein; for Δ5 desaturase assays, the substrate was eicosa-8,11,14-trienoic acid and 2 mg of microsomal protein. Reaction mixtures were incubated with constant shaking at 36 C for 15 min.

The protein was determined by the Lowry procedure using crystalline bovine serum albumin as standard (9). The desaturation reaction was stopped by addition of 2 ml of 10% KOH in ethanol. The fatty acids were recovered by saponification of the incubation mixture (45 min at 82 C), acidification and extraction with petroleum ether (bp 30-40 C). The fatty acids were esterified with methanolic 3 M HCl (1 hr at 64 C).

The analyses were carried out by gas liquid radiochromatography in a Model 893 Packard apparatus equipped with a proportional counter using GP 10% SP 2330 on Chromosorb WAW (100-120 mesh) (10).

The relative radioactivity in [1-^{14}C]substrate and [1-^{14}C]product was determined by measuring the area of

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the counter output peaks by triangulation in the radiochromatograms.

Fatty acid analysis. Total microsomal lipids were extracted using the method of Folch et al. (11), and methyl esters were prepared and analyzed by gas liquid chromatography in a Hewlett-Packard model 5840-A chromatograph equipped with the 5840-A 6C terminal and using a 6-ft column filled with 10% SP 2330 on 100/200 mesh Chromosorb WAW.

Statistical analysis. Data were analyzed by the two-way analysis of variance and Duncan's Multiple Range test.

RESULTS AND DISCUSSION

The effect of environmental temperature observed in rats feeding on CD and HD on Δ6 and Δ5 desaturase activities is shown in Figure 1.

The enzymatic activity of Δ6 desaturase was not modified in those rats on a CD adapted to temperatures of 31 and 12 C when compared to those at 24 C (Fig. 1). These results agree with those of González et al. (6), which showed no significant differences in Δ6 desaturase activity in male rats previously warm-adapted (20–25 days at 31 C) and then shifted to 14 C for 5 days. However, in female rats, an increase in the activity of Δ6 desaturase was observed, and this change was related to estradiol levels (12).

On the contrary, a decrease of the environmental temperature to 12 C evoked an increase of the Δ6 desaturase activity in those rats fed a diet rich in carbohydrates compared to animals maintained at 24 and 31 C.

Peluffo and Brenner (13) found that Δ6 desaturase enzymatic activity in rats kept at 24 C is not modified when isocaloric diets are given in which 5, 15 or 25% of the calories is supplied by proteins. On the other hand, diets with a high carbohydrate content (15,19) decrease Δ6 desaturase enzymatic activity. We may suppose, therefore, that the decrease observed in Δ6 desaturase enzymatic activity at 24 and 31 C as a consequence of the hyperglycidic diet (Fig. 1) is due to its high carbohydrate and not its low protein content. However, the decrease evoked at 12 C was not significant, suggesting that some factors counterbalanced the depressing effect of the hypercarbohydrate diet.

An increase in Δ5 desaturase activity was observed both in rats on a CD and those on a HD kept at 12 C compared to those at 31 C (Fig. 1). There was an increase in Δ5

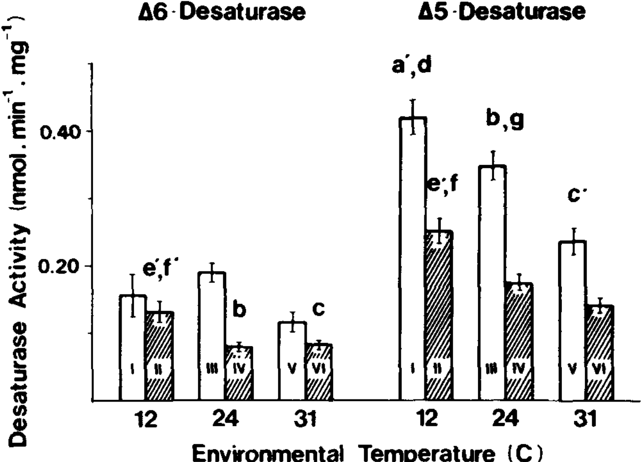

FIG. 1. Effect of environmental temperature on liver microsomal Δ6 and Δ5 desaturase activities in male rats fed control (open bars) and hyperglycidic (shaded bars) diets. Desaturase enzyme assays were carried out as described in Materials and Methods for the six groups of animals described in the text. Results are shown as the mean of 5 rats ± S.E. The type of group is shown within the columns. Letters above the bars indicate significant differences at 1%: a, group I vs group II; b, III vs IV; c, V vs VI; d, I vs V; e, II vs IV; f, II vs VI; and g, III vs V; a', c', e' and f' indicate significant differences at 5%.

TABLE 1

Effect of Environmental Temperature on Liver Microsomal Fatty Acid Composition in Rats Fed Control (CD) and Hyperglycidic (HD) Diets

Fatty acid (%)	12 C		24 C		31 C	
	CD Group I	HD Group II	CD Group III	HD Group IV	CD Group V	HD Group VI
16:0	15.44 ± 0.27^{d}	15.48 ± 0.09$^{g'}$	17.31 ± 0.43^{f}	17.02 ± 0.29	18.84 ± 0.41^{e}	18.22 ± 0.66^{h}
16:1	0.24 ± 0.15^{d}	0.42 ± 0.17	0.62 ± 0.22$^{b'}$	1.24 ± 0.13^{i}	1.60 ± 0.22^{e}	1.64 ± 0.14^{h}
18:0	24.08 ± 0.34	24.82 ± 0.29	22.25 ± 0.96	23.16 ± 0.60^{i}	20.52 ± 1.02^{e}	19.64 ± 0.78^{h}
18:1	8.66 ± 0.23	9.12 ± 0.25	9.88 ± 0.24	10.20 ± 0.37	9.86 ± 0.63	11.12 ± 0.60^{h}
18:2n-6	13.42 ± 0.47$^{a'}$	15.20 ± 0.33	13.78 ± 0.54^{b}	15.58 ± 0.21	13.90 ± 0.43	15.94 ± 0.71$^{c'}$
20:3n-6	0.78 ± 0.04$^{a'}$	1.20 ± 0.05	1.05 ± 0.09^{b,f}	1.50 ± 0.07$^{i'}$	1.58 ± 0.11^{e}	1.86 ± 0.22^{h}
20:4n-6	31.72 ± 0.41^{a}	28.15 ± 0.41^{g}	31.82 ± 1.20^{b,f}	26.64 ± 0.33^{i}	28.54 ± 0.49^{e}	26.44 ± 0.63$^{c',h}$
22:5n-3	0.98 ± 0.10$^{a'}$	1.36 ± 0.13	1.10 ± 0.11	1.36 ± 0.11	0.90 ± 0.08	1.28 ± 0.10$^{c'}$
22:6n-3	4.58 ± 0.29	4.18 ± 0.07^{g}	4.55 ± 0.16$^{b'}$	3.24 ± 0.31$^{i'}$	3.60 ± 0.33	3.84 ± 0.26
20:4n-6/18:2n-6	2.364 ± 0.113^{a}	1.852 ± 0.067	2.309 ± 0.178^{b,f}	1.710 ± 0.044	2.053 ± 0.099^{e}	1.659 ± 0.113$^{c'}$
Unsaturation index	1.866 ± 0.013^{a}	1.788 ± 0.010^{g}	1.864 ± 0.014^{b,f}	1.697 ± 0.015^{i}	1.764 ± 0.012^{e}	1.752 ± 0.014$^{h'}$

Fatty acid composition was determined by gas liquid chromatography as described in Materials and Methods for the 6 groups of animals described in the text. Only main fatty acids were considered. Results are shown as mean of 5 rats ± S.E. Letters indicate significant differences at 1%: *a*, group I vs group II; *b*, III vs IV; *c*, V vs VI; *d*, I vs III; *e*, I vs V; *f*, III vs V; *g*, II vs IV; *h*, II vs VI; *i*, IV vs VI; *a'*, *b'*, *c'*, *f'*, *g'*, *h'* and *i'* indicate significant differences at 5%.

desaturase activity only in HD rats kept at 12 C compared to those at 24 C. The highest activity was observed in the animals kept at the lowest temperatures.

The Δ5 desaturase enzymatic activity decreased in animals on a HD compared to rats on a CD, irrespective of environmental temperature.

Although we do not know the mechanism of the HD effect, we do know that epinephrine (16,17) and cAMP (17) evoke a decrease of fatty acid desaturase activity. Moreover, glucocorticoids also induce a decrease of desaturase activity (18). However, Jeffcoat and James (19) were unable to detect a decrease of Δ5 desaturase activity when feeding rats a diet rich in sucrose.

Fatty acid composition of lipids was also studied, since changes in microsomal desaturation can be detected in microsomal lipids (20). In this respect, animals fed a HD and kept at 12 C show a higher unsaturation index than animals kept at 24 and 31 C. This could be related to an increase of Δ6 and Δ5 desaturation. The effect of the HD is shown in Table 1. In all animals, the decrease in the 20:4n-6/18:2n-6 ratio is in accord with a decrease of Δ6 and Δ5 desaturase activities (Fig. 1). However, in those rats kept at 12 C, no modification in Δ6 desaturase activity was observed.

Rats kept on CD and HD at 12 C showed a decrease in 20:3n-6 and an increase in 20:4n-6 compared to those groups at higher temperatures; these changes could be attributed to the increase of Δ5 desaturase activity caused by low temperature.

In conclusion, results suggest that cold evokes an increase of Δ6 and Δ5 desaturase activities when these enzymes are suppressed by dietary factors, such as a hyperglycidic diet.

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1-Acyl-2-acetyl-*sn*-glycero-3-phosphocholine from Stimulated Human Polymorphonuclear Leukocytes

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1-Acyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-acyl-2-acetyl GPC) was found in the fraction of platelet-activating factor obtained from stimulated human polymorphonuclear leukocytes (PMN). The amount of 1-acyl-2-acetyl GPC obtained from 1 × 10^7 PMN stimulated with ionophore A23187 at 37 C for 15 min ranged from 8 to 56 pmol (32 ± 10 pmol, mean ± standard error; n = 4). The main species was 16:0 palmitoyl (17 ± 5 pmol), followed by 18:0 stearoyl (8 ± 3 pmol) and 18:1 oleoyl (7 ± 3 pmol).

Although the physiological significance is unknown, 1-acyl-2-acetyl GPC was always detected when 1-alkyl-2-acetyl GPC was detected.
Lipids 22, 285–287 (1987).

The platelet-activating factor (PAF) generated by human polymorphonuclear leukocytes (PMN) was identified by selected ion monitoring (SIM) and fast atom bombardment mass spectrometry (FAB-MS) as 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-alkyl-2-acetyl GPC), composed mainly of 16:0 1-hexadecyl-2-acetyl species (1–3). [The abbreviation PAF refers to biological activity. To avoid confusion, each molecular species of 1-alkyl-2-acetyl GPC is termed by its systematic name and each species of 1-acyl-2-acetyl GPC by its trivial name.] At the same time, it was shown by mass spectrometry that 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-acyl-2-acetyl GPC), in which a fatty acyl group is substituted for a long chain alcohol at the *sn*-1 position, is generated by stimulated rabbit PMN and that its predominant species was 16:0 palmitoyl, followed by 18:1 oleoyl and 18:0 stearoyl (4).

A well-known potential of 1-acyl-2-acetyl GPC is its ability to compete with 1-alkyl-2-acetyl GPC for acetylhydrolase (5,6). Recently, cell-associated PAF generated by PMN stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP) was shown to act as a feedback inhibitor of PAF biosynthesis (7).

In this work, we extended our previous studies (4) by SIM examination to the PAF fraction from stimulated human PMN, and we also examined the potential role of 1-acyl-2-acetyl GPC.

MATERIALS AND METHODS

Chemicals. The chemotactic peptide FMLP was purchased from the Protein Research Foundation (Osaka, Japan). Calcium ionophore A23187 was obtained from Calbiochem-Behring (San Diego, California). 1-Acyl-2-acetyl GPC, a mixture of the palmitoyl and stearoyl species (2:1, w/w), was synthesized by mixing acetic anhydride and the respective lysocholine phosphoglycerides (4). Sources of other chemicals were as described in our recent publications (4,8,9).

Isolation of PMN and platelets. The procedure for isolation of human PMN, involving dextran sedimentation, Ficoll-paque gradient centrifugation and hypotonic treatment to lyse erythrocytes, was described previously (8). Preparation of washed rabbit platelets essentially following the method of Pinckard et al. (10) has been described previously in further detail (9). Human platelet-rich plasma (PRP) was prepared by centrifuging citrated blood at 225 × g for 15 min at 25 C.

SIM analysis of molecular species. Human PMN from 100 ml of citrated blood were resuspended at a concentration of 6 × 10^6 cells/ml in Tyrode's solution containing 0.25% bovine serum albumin (BSA) and 1.3 mM $CaCl_2$. The procedures used to activate PMN and to extract, isolate and derivatize 1-alkyl-2-acetyl GPC and 1-acyl-2-acetyl GPC were as described previously (4). In the extraction step, 200 ng of 1-hexadecyl-2-acetyl-d_3 GPC and 100 ng of 1-heptadecanoyl-2-acetyl-d_3 GPC were added as internal standards. Lipids of interest were located from their positions relative to the standards, which were located by the rhodamine method (4). Three types of ions, $(CH_3CO+74)^+$, $(alkyl+130)^+$ and $(M-57)^+$, were monitored. Other conditions for SIM were as described previously (1).

Effect of 1-acyl-2-acetyl GPC on the production of PAF by PMN. Samples of 5 ml of human PMN (5 × 10^6 PMN/ml Tyrode's gelatin solution, pH 7.2, containing 1.3 mM $CaCl_2$) were preincubated with 1 × 10^{-10} M 1-acyl-2-acetyl GPC for 1 min at 37 C and then were stimulated with 1 × 10^{-6} M FMLP for 2.5 min. The reaction mixtures were transferred to other glass tubes, and total lipids were extracted by the systems of Bligh and Dyer (11). The lipid extract was suspended in 2.5 mg BSA/ml saline. The amount of PAF equivalents generated by PMN was measured as described (12) by aggregation of washed rabbit platelets, except for no addition of cycloxygenase inhibitor and ADP scavenger. 1-Hexadecyl-2-acetyl GPC was used as a standard, because human PMN produce this species predominantly (1–3).

RESULTS AND DISCUSSION

Simultaneous presence of 1-alkyl-2-acetyl GPC and 1-acyl-2-acetyl GPC. The presence of both 1-alkyl-2-acetyl GPC and 1-acyl-2-acetyl GPC in stimulated human PMN was demonstrated by SIM analysis of PAF samples before separation of the two types by thin layer chromatography (TLC). Upon monitoring at m/z 117, six peaks were recorded with retention times of 0′53″, 1′05″, 1′20″, 1′38″, 2′06″ and 2′36″ (Fig. 1). From comparison of their retention times with those of standards, as well as monitoring at m/z $(M-57)^+$, six molecular species were identified in the four main peaks: 1-hexadecyl-2-acetyl GPC (m/z 415, 1′05″), 1-palmitoyl-2-acetyl GPC (m/z 429, 1′20″), 1-octadecenyl-2-acetyl GPC (m/z 441, 1′36″), 1-octadecyl-2-acetyl GPC (m/z 443, 1′43″), 1-oleoyl-2-acetyl GPC (m/z 455, 2′00″) and 1-stearoyl-2-acetyl GPC (m/z 457, 2′05″), in order of their elutions. However, as stressed previously (4), it is necessary to consider the presence of odd-numbered 1-alkyl-2-acetyl GPC on the analysis of even-numbered 1-acyl-2-acetyl GPC. To this end, the spot

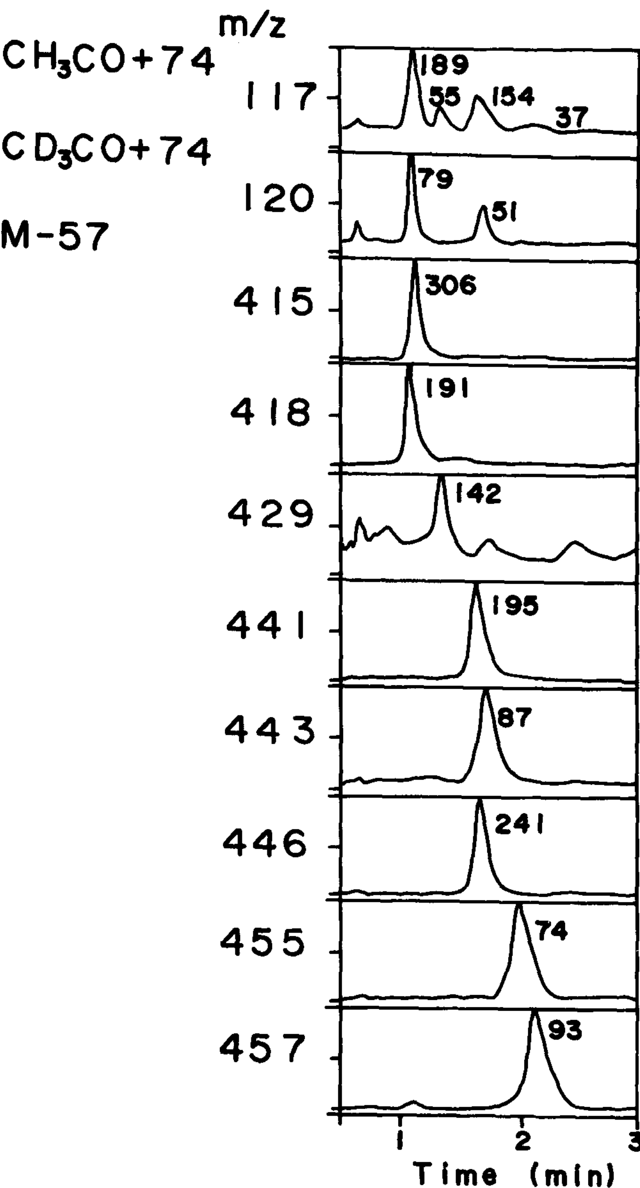

FIG. 1. Selected ion monitoring (SIM) trace of platelet-activating factor (PAF) sample from stimulated human polymorphonuclear leukocytes (PMN). Human PMN (1.2×10^8 cells) were challenged with 2.5×10^{-6} M ionophore A23187. The PAF fraction was purified from the cells plus medium and derivatized. SIM analyses were performed separately for the acetyl group and groups with molecular weights of the 1-alkyl-2-acetyl and 1-acyl-2-acetyl GPCs. The signals at m/z 418 and m/z 446 were those of ions of internal standards. Peak areas are shown on the right of each peak.

corresponding to 1-alkyl-2-acetyl-3-*tert*-butyldimethylsilyl (*t*-BDMS) glycerol was purified by TLC in hexane/diethylether (9:1, v/v) (1). By monitoring at m/z 117, the peaks at 1′20″ and 2′06″ almost disappeared, leading to the conclusion that the quantities of odd-numbered 1-alkyl-2-acetyl GPC are minute. Accordingly, simultaneous quantitations were performed using calibration curves of the $(M\text{-}57)^+$ ion of 1-alkyl-2-acetyl GPC (1,9) and 1-acyl-2-acetyl GPC (4). The amounts of 1-alkyl-2-acetyl GPC in 1×10^7 PMN (n = 4) were 94 ± 36 pmol (mean ± standard error) of 1-hexadecyl-2-acetyl GPC, 37 ± 13 pmol of 1-octadecenyl-2-acetyl GPC and 29 ± 12 pmol of 1-octadecyl-2-acetyl GPC. In contrast, one-fifth of this amount of 1-acyl-2-acetyl GPC was detected. The main species was 1-palmitoyl-2-acetyl GPC (17 ± 5 pmol), followed by 1-stearoyl-2-acetyl GPC (8 ± 3 pmol) and 1-oleoyl-2-acetyl GPC (7 ± 3 pmol). A close correlation was found between the amounts of 1-alkyl-2-acetyl GPC and 1-acyl-2-acetyl GPC. In many experiments, including the present one, PAF was detected with iodine vapor. This procedure was found to affect the quantitation of unsaturated species of both types (4), so saturated species were represented. The amounts of 1-hexadecyl- (71 ± 19 pmol) plus 1-octadecyl-2-acetyl GPC (21 ± 7 pmol) per 1×10^7 PMN were plotted on the x-axis and those of 1-palmitoyl- (15 ± 3 pmol) plus 1-stearoyl-2-acetyl GPC (5 ± 2 pmol) on the y-axis. PAF fractions 4 to 8 were detected by the iodine method and quantitated with 1-palmitoyl-2-acetyl-d_3-GPC as an internal standard. The regression equation was $Y = 0.17\ X + 4.91$ with a correlation coefficient of $r = 0.989$ (n = 8). This is quite in contrast to the regression equation of rabbit PMN, $Y = 0.66\ X + 11.61$, $r = 0.940$ (4).

There is no direct method available for measurements of 1-acyl-2-acetyl GPC and 1-alkyl-2-acetyl GPC in mixtures of the two. The peak of intact 1-palmitoyl-2-acetyl GPC overlapped that of 1-pentadecyl-2-acetyl GPC on high performance liquid chromatography (HPLC) (13), and peaks of derivatives of 1-palmitoyl-2-acetyl species overlapped those of 1-heptadecyl-2-acetyl species on GC with packed column (4). In fact, Weintraub detected 1-palmitoyl-2-acetyl GPC by FAB-MS as one component in the peak separated by reversed-phase HPLC, in which 1-pentadecyl, 1-hexadecenyl and 1-octadidecenyl-2-acetyl GPC were also detected (14). So, it is necessary to separate these types of PAF before analyses of molecular species. Ramesha and Pickett analyzed the molecular species of PAF from ionophore A23187-stimulated rat peritoneal PMN without their separation (15). They used capillary GC/negative ion chemical ionization mass spectrometry of the molecular ion, $(M)^-$, for PAF determination (16), and detected three isometric peaks of 1-heptadecyl-2-acetyl GPC and also 1-nonadecyl-2-acetyl GPC. Judging from the retention times and mass numbers of these fractions, they could have contained 1-acyl-2-acetyl GPC in addition to odd-numbered 1-alkyl-2-acetyl GPC. Assuming that these fractions consisted entirely of 1-acyl-2-acetyl GPC, the percentage of this type was at most 2.2%, as calculated simply as the sum of the peak areas. Thus the contribution of 1-acyl-2-acetyl GPC seems to be minute in rat PMN.

Biological effect of 1-acyl-2-acetyl GPC. The biological activity of 1-acyl-2-acetyl GPC was assessed by measuring its ability to elicit platelet aggregation or to inhibit aggregation of human PRP induced by 1-alkyl-2-acetyl GPC in an aggregometer (Nikko Hematracer, PAT-4A). The platelets were stirred at 1000 rpm, and changes in light transmission were recorded with time. Experiments were completed within 1 hr after PRP preparation (17). 1-Acyl-2-acetyl GPC is a weak agonist to human PRP, as it is to washed rabbit platelets (18). Even at 2×10^{-6} M, it induced a very weak primary aggregating response. In contrast, 1-alkyl-2-acetyl GPC elicited an immediate and irreversible aggregating response of human PRP at a concentration of 1×10^{-7} M. At a concentration of 6×10^{-8} M, it caused biphasic aggregation, and at a lower

concentration, it caused no secondary aggregation, reversible aggregation only being noted. Preincubation of 1-acyl-2-acetyl GPC with PRP or simultaneous addition of 1-acyl-2-acetyl GPC at a final concentration of 2×10^{-6} M with 6×10^{-8} M 1-alkyl-2-acetyl GPC had no inhibitory or stimulatory effect on biphasic aggregation elicited by the latter.

We have demonstrated that most of the 1-alkyl-2-acetyl GPC generated by ionophore A23187-stimulated PMN remained cell-associated (1,8), and this was also observed when opsonized zymosan or FMLP was employed as a stimulus (19). We found that 1-acyl-2-acetyl GPC was also mainly cell-associated. When PMN treated with ionophore A23187 were separated by rapid centrifugation, 31 ± 8 pmol ($n = 2$) saturated 1-acyl-2-acetyl GPC per 1×10^7 PMN remained in a cell-associated form, while 4 ± 2 pmol ($n = 3$) was detected in the supernatant. At the same time, 77 ± 40 pmol ($n = 2$) of saturated 1-alkyl-2-acetyl GPC was recovered in the cell pellet and 10 ± 4 pmol ($n = 3$) in the supernatant fraction. Ludwig et al. demonstrated recently that this cell-associated PAF acts as a feedback inhibitor of PAF biosynthesis (7). Accordingly, we examined the possible role of 1-acyl-2-acetyl GPC as an inhibitor of the PAF synthesis by PMN.

PAF production, measured 2.5 min after stimulation, was greatest on stimulation with 1×10^{-6} M FMLP. The amount of PAF produced by 5×10^6 PMN was 356 ± 121 fmol with a range of 140 to 700 fmol ($n = 5$). The high degree of variability of PAF production by human PMN was also observed by Ludwig et al. (20). Assuming that one-fifth of this amount is acyl type and 90% of this is cell-associated, it is calculated to be 60 fmol per 5×10^6 PMN. Next, about 10–20% of exogenously added PAF associated with PMN (21). Accordingly, we preincubated PMN with 1×10^{-10} M 1-acyl-2-acetyl GPC for 1 min. Roughly, 10–20 fmol is expected to be associated with 5×10^6 PMN. They were stimulated with 1×10^{-6} M FMLP for 2.5 min. The amount of PAF produced by 5×10^6 PMN was 324 ± 105 fmol with a range of 80 to 620 fmol ($n = 5$). So preincubation of PMN with 1-acyl-2-acetyl GPC was inhibitory, but a special role of acyl-type PAF as feedback inhibitor would be ruled out at the initial phase of PAF biosynthesis.

Finally, the physiological significance of 1-acyl-2-acetyl GPC is still obscure. However, as stressed by Hanahan in a recent review (22), it does not mean that this type of PAF is not metabolically important.

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Acyltransferases in Subcellular Fractions of Developing Seeds of Rape (*Brassica napus* L.)

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Acyltransferase activities responsible for the transfer of oleoyl moieties from oleoyl-CoA to various lipids have been examined in subcellular fractions of developing seeds of rape, *Brassica napus* L. In the absence of exogenous acyl acceptors, the microsomal and oil body fractions transferred oleoyl moieties mostly to phosphatidylcholines and phosphatidic acids, although there was substantial incorporation of the oleoyl moieties into monoacylglycerols, diacylglycerols and triacylglycerols. The soluble (150,000-g supernatant) fraction incorporated oleoyl moieties mainly into the neutral lipids (monoacylglycerols and diacylglycerols) and also exhibited a relatively high acyl-CoA hydrolase activity. In the presence of the exogenous acyl acceptors, lysophosphatidylcholine and lysophosphatidic acid, both microsomal and oil body fractions transferred most of the oleoyl moieties to phosphatidylcholines and phosphatidic acids, respectively. Other lysophospholipids, such as lysophosphatidylethanolamine and lysophosphatidylinositol, were not very effective as acyl acceptors, nor were glycerol-3-phosphate, monoacylglycerols or diacylglycerols. In contrast, the soluble fraction showed little or no stimulation of acyltransfer in the presence of exogenous lysophospholipids but was able to utilize, to some extent, exogenous monoacylglycerols and diacylglycerols as acyl acceptors.
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Triacylglycerol biosynthesis is believed to occur in oilseeds via the Kennedy pathway (1), as in animal tissues. In many oilseeds, however, acyl groups are also channeled toward a phosphatidylcholine pool, which is probably the substrate for the desaturase(s) involved in the formation of polyunsaturated acyl moieties (2,3). The polyunsaturated acyl moieties then return to the Kennedy pathway as diacylglycerols (4–6) and/or acyl-CoA derivatives (6–8). The relative rates of the following reactions in oilseeds remain to be determined, and the involvement of these pathways in the formation of triacylglycerols is the subject of some controversy (3–6,9,10):

$$\text{Acyl-CoA} + \text{Lysophosphatidylcholine} \rightleftharpoons \text{Phosphatidylcholine} + \text{CoASH}$$
$$\text{1,2-Diacylglycerol} + \text{CDP-choline} \rightleftharpoons \text{Phosphatidylcholine} + \text{CMP}$$

Equally unclear is the subcellular localization of the various enzymes involved in triacylglycerol biosynthesis. Most studies have previously been concentrated on the so-called "microsomal" fraction, i.e., the membrane fraction sedimenting between 10,000 and 100,000 g. This membrane fraction contains most of the enzymes involved in triacylglycerol biosynthesis, but it may not be the only, or even the major, site of each of the constituent enzymes involved. The oil body fraction has been reported as a major site of triacylglycerol formation in seeds of crambé (11), mustard (12) and castor (13) and of wax ester formation in jojoba seeds (14). The high (19%, w/w) protein content reported for crambé oil bodies (11) contrasts with that for oil bodies from peanut (0.2%, w/w) and from linseed and safflower (2.5%, w/w) (15). The finding that linseed and safflower oil bodies contain only four major polypeptides of 14–18 kDa casts some doubt on the biosynthetic capacity of these organelles (3). The biosynthetic capacity of the soluble fraction from developing oilseeds has hitherto been neglected. Recently, however, a soluble glycerol-3-phosphate acyltransferase has been purified from cocoa seeds (16), and developing mustard seeds have been shown to exhibit soluble acyltransferase activities (12).

In the present study, the acyltransferase activities leading to triacylglycerol biosynthesis were examined in developing seeds of oilseed rape, which is a major oilseed crop in northern Europe, Asia and Canada. The subcellular distribution of acyltransferases has been examined in microsomal, oil body and soluble fractions obtained from the seed homogenate.

MATERIALS AND METHODS

Materials. Developing seeds of rape, var. Jet Neuf, were harvested at a period of high triacylglycerol formation, i.e., 4–5 weeks after flowering, and immediately frozen to −80 C. The seeds used in the present study were a gift of A. R. Slabas (Unilever Research, Sharnbrook, Welwyn, Bedfordshire, United Kingdom). [1-^{14}C]Oleoyl-CoA (2.068 KBq.nmol^{-1}) was purchased from Amersham-Buchler (Braunschweig, Federal Republic of Germany). Oleoyl-CoA, glycerol-3-phosphate, 1-oleoylglycerol, dioleoylglycerols (85% *sn*-1,3 and 15% *sn*-1,2[2,3] isomers), 1-acyllysophospholipids (oleoyllysophosphatidylcholine, oleoyllysophosphatidic acid, palmitoyllysophosphatidylethanolamine and lysophosphatidylinositol [from soybean]), and CoASH were obtained from Sigma-Chemie (Muenchen, Federal Republic of Germany). All other reagents and adsorbents were from E. Merck (Darmstadt, Federal Republic of Germany). Lipid standards for thin layer chromatography (TLC) were either from Sigma-Chemie or Applied Science Laboratories (State College, Pennsylvania).

Isolation of subcellular fractions. All operations were carried out at 4 C. Frozen seeds (10 g) were ground in a mortar in a total of 40 ml isolation buffer consisting of 50 mM HEPES-KOH, pH 7.4, 1 mM EDTA, 1 mM DTT and 0.5 M sucrose. The brei was filtered through one layer of Miracloth to yield the homogenate fraction, which was centrifuged at 12,000 × g for 20 min to yield floating oil body (O_1), supernatant (S_1) and pellet (P_1) fractions. $MgCl_2$ was added to the supernatant to a final concentration of 50 mM to precipitate the microsomal membranes. The microsomal fraction was then sedimented at 20,000 × g for 15 min. The resulting supernatant (S_2) was subjected to further centrifugation at 150,000 × g for 2 hr to obtain a soluble protein fraction. O_1 was dispersed in isolation

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buffer by gentle passage through a ground-glass homogenizer and centrifuged at 20,000 × g for 15 min to yield a washed oil body fraction. This procedure was repeated three times until no further membranous material sedimented and a thick creamy washed oil body fraction was obtained. The washed oil body and the microsomal fractions were resuspended in a medium consisting of 50 mM HEPES-KOH, pH 7.4, 1 mM DTT and 10 mM $MgCl_2$.

Incubations. Aliquots of subcellular fractions corresponding to 0.2–0.4 mg protein were incubated in a total volume of 500 µl of a medium consisting of 50 mM HEPES-KOH, pH 7.4, 1 mM ATP, 0.3 mM CoASH, 5 mM $MgCl_2$ and 1 mM 2-mercaptoethanol. Incubations were started by adding 3.7 KBq [1-^{14}C]oleoyl-CoA at a final concentration of 20 nmol oleoyl-CoA per assay. When lysophospholipids or mixtures of mono- and diacylglycerols were used as exogenous acyl acceptors, 100 nmol of these lipids were added as sonicated dispersions as described elsewhere (17). Incubations were carried out at 23 C in open glass tubes with shaking and were terminated by the addition of 10 ml chloroform/methanol (2:1, v/v) to produce a monophasic extract. To this extract was added 2 ml of an aqueous solution of 0.1% acetic acid containing 0.7% NaCl, which resulted in partitioning of all water-soluble constituents, including acyl-CoA derivatives, into the upper aqueous phase. The lower phase containing the chloroform-soluble lipids was recovered and analyzed.

Lipid and protein analysis. Methods for analysis of chloroform-soluble lipids and detection of acyl-CoA in the aqueous phase after partitioning of the lipids were identical to those described previously (17,18). In addition, the following systems for TLC were employed.

To detect monoacylglycerols, the total lipids were applied to layers of Silica Gel H, and the chromatoplates were developed twice with diethyl ether to a height of 2 cm from the origin. Upon subsequent development of the chromatoplates up to the top with hexane/diethyl ether/acetic acid (70:30:1, v/v/v), the monoacylglycerols, diacylglycerols, unesterified fatty acids and triacylglycerols were separated from each other and from the mixture of phospholipids and glycolipids that remained at the origin.

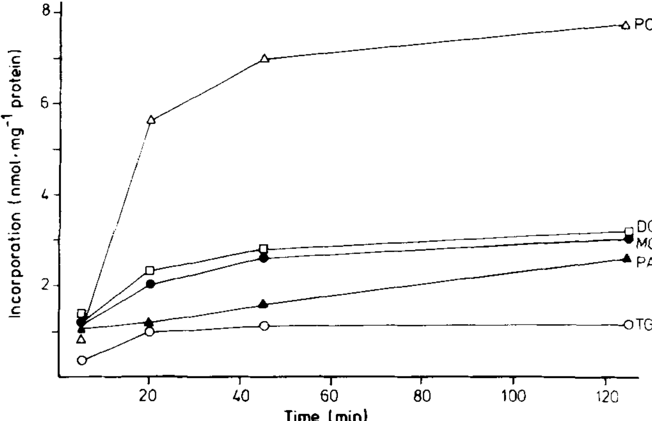
The identity of individual classes of phospholipids and glycolipids was established by TLC on Silica Gel H using two different solvent systems, i.e., chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v/v/v/v) and chloroform/methanol/concentrated ammonium hydroxide (65:35:5, v/v/v). Monoacylglycerols were also separated on boric acid-impregnated Silica Gel H chromatoplates for the purpose of positional analysis of acyl groups (19).

Protein content of the subcellular fractions was determined according to an established method (20).

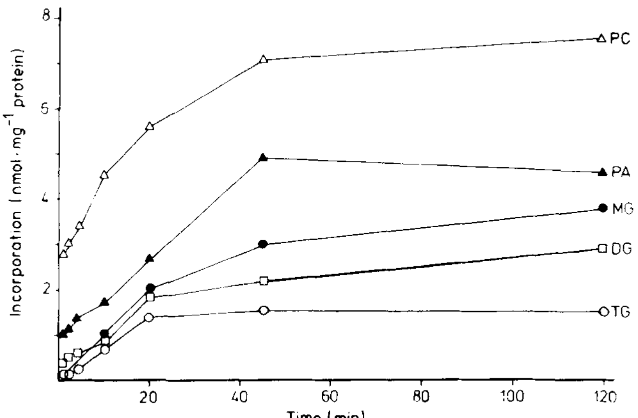
RESULTS AND DISCUSSION

The time course of metabolism of [1-^{14}C]oleoyl-CoA by microsomal, oil body and soluble fractions from developing rapeseed are shown in Figure 1. All of these incubations were performed in the absence of exogenous acyl acceptors. As previously shown for safflower (21–23), sunflower (7,24), crambé (11) and other oilseeds (8,25) the microsomal fraction rapidly incorporated oleoyl moieties into phosphatidylcholines (Fig. 1). The oil body fraction

A: Microsomes



B: Oil bodies



C: Soluble fraction

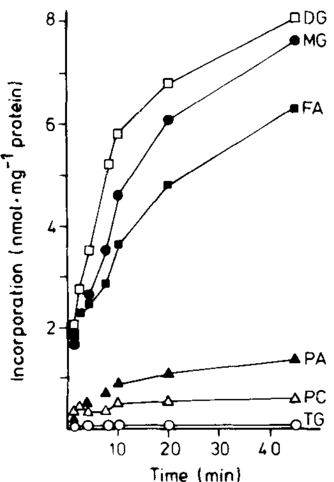


FIG. 1. Time course of incorporation of oleoyl moieties into lipids upon incubation of subcellular fractions of developing rapeseed with [1-^{14}C]oleoyl-CoA. Microsomes, oil body and a soluble fraction from developing rapeseed were incubated for different periods with 3.7 KBq [1-^{14}C]oleoyl-CoA (20 nmol) and the incorporation of oleoyl moieties (nmol.mg^{-1} protein) into various lipids was determined as described in the text: ●, monoacylglycerols; □, diacylglycerols; ○, triacylglycerols; ■, unesterified fatty acids; ▲, phosphatidic acids; △, phosphatidylcholines. All values are means of at least two independent experiments.

also incorporated most of the oleoyl moieties into phosphatidylcholines, although there was a rapid incorporation of oleoyl moieties into phosphatidic acids as well (Fig. 1). This contrasts with mustard oil bodies, which incorporated three times as much of the oleoyl moieties from oleoyl-CoA into phosphatidic acids than into phosphatidylcholines in one hour (12). The soluble fraction incorporated relatively little of the oleoyl moieties into any of the polar lipids (Fig. 1). None of the subcellular fractions of developing rapeseed incorporated significant amounts of oleoyl moieties into the glycolipids.

The relatively high level of incorporation of oleoyl moieties into mono- and diacylglycerols by all subcellular fractions (Fig. 1) was somewhat surprising. It has been reported that monoacylglycerols were labeled from [U-^{14}C]glycerol in crambé oil bodies (11) and from [1-^{14}C]-glycerol-3-phosphate in microsomes from safflower (26) and avocado (27) and in cucumber cotyledon homogenates (28), but in most cases the extent of labeling was relatively low. In a recent study, however, relatively high proportions of labeled monoacylglycerols were formed from [1-^{14}C]oleoyl-CoA in homogenate, oil body and soluble fractions from developing mustard seeds (12). Monoacylglycerols migrate only a short distance from the origin of the chromatograms in most commonly used solvent systems for TLC of neutral lipids, and are therefore poorly resolved from the polar lipid band remaining at the origin. In the present study, the monoacylglycerols were allowed to migrate 2 cm from the origin together with the other neutral lipids by double development with diethyl ether, which was followed by fractionation of the neutral lipids with hexane/diethyl ether/acetic acid (70:30:1, v/v/v). The monoacylglycerols were further analyzed by chromatography on boric acid-impregnated thin layer plates. This showed that essentially all of the oleoyl moieties were incorporated into the *sn*-1(3) position of monoacylglycerols.

The nature of the diacylglycerols labeled with [1-^{14}C]-oleoyl moieties that were formed in the various fractions was determined by TLC on Silica Gel H. The ratio of labeled 1,2-(2,3) diacylglycerols to 1,3-diacylglycerols ranged from 1:2 to 2:1 in microsomes, 1:1 to 3:1 in oil bodies and 2:1 to 3:1 in the soluble fraction.

In addition to actively incorporating oleoyl moieties into mono- and diacylglycerols, the soluble fraction contained a considerable thioesterase activity, as evidenced by the large proportion of radioactive oleic acid formed (Fig. 1). Similar observations have been reported for soluble fractions from developing seeds of safflower (21) and mustard (12). Thioesterase activities were also found in the microsomal and oil body fractions in the present study, but these were at much lower levels than that of the soluble fraction (data not shown).

Since all three subcellular fractions of developing rapeseed were able to efficiently transfer oleoyl moieties onto endogenous complex lipids, it was of interest to investigate their capacity to utilize exogenous acyl acceptors in the presence of oleoyl-CoA. In Table 1, the effect of a wide range of acyl acceptors on the incorporation of oleoyl moieties from oleoyl-CoA is shown for each subcellular fraction. In the case of the microsomal and oil body fractions, the most dramatic results were obtained with lysophosphatidylcholine and lysophosphatidic acid, both of which caused a greatly increased uptake of oleoyl moieties from oleoyl-CoA and also the specific channelling of these moieties into the corresponding diacylphospholipids. This was not the case with the other two exogenous lysophospholipids used here, i.e., lysophosphatidylethanolamine and lysophosphatidylinositol. Therefore,

TABLE 1

Effect of Exogenous Acyl Acceptors on the Incorporation of [1-^{14}C]Oleoyl-CoA by Subcellular Fractions of Developing Rapeseed

		Incorporation of oleoyl moieties into acyl lipids (nmol.mg^{-1} protein)[a]						
Fraction	Acyl acceptor	MG	DG	TG	PA[b]	PC[b]	PE[b]	PI
Microsomes	None	2.0	2.3	1.0	1.2	5.6	0.3	0.3
	Lysophosphatidylcholine	0.3	0.4	0.4	0.3	24.2	0.2	0.1
	Lysophosphatidylethanolamine	1.7	2.0	0.6	2.9	3.7	2.2	0.2
	Lysophosphatidylinositol	1.9	1.6	0.5	1.1	2.8	0.6	0.2
	Lysophosphatidic acid	2.1	3.3	0.5	11.8	4.7	4.6	0.2
	Glycerol-3-phosphate	1.8	2.0	0.5	1.7	2.9	0.5	0.2
Oil bodies	None	2.0	1.9	1.4	2.7	5.5	2.0	0.7
	Lysophosphatidylcholine	1.7	2.0	2.2	8.5	17.4	3.6	0.8
	Lysophosphatidylethanolamine	1.7	1.8	0.4	2.7	4.8	2.7	0.8
	Lysophosphatidylinositol	2.2	2.1	0.6	2.3	4.8	1.3	1.0
	Lysophosphatidic acid	1.8	3.0	2.5	12.3	2.6	5.9	0.4
	Glycerol-3-phosphate	3.2	1.9	1.5	4.5	3.4	0.5	0.5
	Mono- + diacylglycerols	3.0	2.3	1.7	3.8	4.2	0.4	0.6
Soluble fraction	None	3.5	4.8	tr[c]	0.9	0.3	0.1	tr
	Lysophosphatidic acid	4.5	6.3	tr	0.5	0.6	tr	tr
	Glycerol-3-phosphate	5.7	5.6	tr	0.8	0.2	0.1	tr
	Mono- + diacylglycerols	5.5	5.7	tr	0.4	0.6	tr	tr

MG, monoacylglycerols; DG, diacylglycerols; TG, triacylglycerols; PA, phosphatidic acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PI, phosphatidylinositols.

[a]Microsomes, oil bodies and a soluble fraction from developing rapeseed were incubated for 20 min with 3.7 KBq [1-^{14}C]oleoyl-CoA (20 nmol) in the presence of various acyl acceptors and the incorporation of oleoyl moieties into various lipids determined as described in Materials and Methods. Values are the means of at least two independent experiments.

[b]Including the corresponding lysophospholipids.

[c]Traces.

the acyltransferase(s) responsible for transfer of oleoyl moieties to phosphatidylcholines and phosphatidic acids in microsomes and oil bodies of rapeseed appear to have a far greater degree of specificity for acyl acceptors than those present in microsomes from safflower seeds (29).

The addition of either glycerol-3-phosphate or a mixture of mono- and diacylglycerols was expected to result in an increase in the flow of intermediates through the Kennedy pathway (1) and therefore to increase [1-^{14}C]-oleoyl-CoA incorporation, as has been observed in other oilseeds (7,10,12,30). In the case of microsomes and oil bodies from rapeseed, however, these exogenous acyl acceptors caused a slight but definite decrease in incorporation of oleoyl moieties (Table 1). Despite the overall decrease in incorporation, the microsomes from rapeseed incorporated more oleoyl moieties into phosphatidic acids in the presence of glycerol-3-phosphate, while the incorporation into phosphatidylcholines was halved (Table 1). This is consistent with an increased flux through the earlier part of the Kennedy pathway and an associated decrease in acyl transfer to phosphatidylcholines, but there was no evidence of increased synthesis of di- or triacylglycerols in the presence of exogenous glycerol-3-phosphate. This implies that there was no increase in the flow of acyl groups through the later part of the Kennedy pathway.

As compared to microsomes, the oil bodies from rapeseed incorporated higher proportions of oleoyl moieties into neutral lipids in the presence of exogenous glycerol-3-phosphate (Table 1). There was also a sizeable increase in the incorporation of oleoyl moieties into phosphatidic acids. Therefore, the oil body fraction from developing rapeseed represents a better source (on a mg^{-1} protein basis) of Kennedy pathway activities as a whole than does the microsomal fraction.

The soluble fraction was deficient in lysophospholipid acyltransferase(s) and in diacylglycerol acyltransferase (Table 1). Thus, even when diacylglycerol formation was stimulated by exogenous acyl acceptors, such as lysophosphatidic acid and mono- plus diacylglycerols, no more than trace amounts of triacylglycerols were detected. Concomitant with the stimulation of incorporation of oleoyl moieties into diacylglycerols, an increase in monoacylglycerol labeling was observed (Table 1). The latter could have been formed by two possible reactions, i.e., acyl hydrolysis of diacylglycerols or cleavage of the phosphate group by a lysophosphatidic acid phosphatase. Germinating rapeseed is a good source of acylhydrolase activity (31), which has been localized in both the microsomal (32) and oil body (33) fractions, but it is not known whether developing seeds, such as those used in the present study, contain any of this activity. Acyl hydrolysis by nonspecific hydrolase(s) is another possibility, and the soluble fraction from rapeseed did contain an acyl-CoA hydrolase activity (Fig. 1), which may be due to such a nonspecific hydrolase. If the monoacylglycerols were generated instead by a lysophosphatidic acid phosphatase, this could have been due to overlapping specificity of phosphatidic acid phosphatase, which is a Kennedy pathway enzyme responsible for the formation of diacylglycerols. This latter activity has been detected in both microsomal and soluble fractions from castor bean endosperm (34). A monoacylglycerol kinase, which catalyzes the reaction

$$\text{monoacylglycerol} + \text{ATP} \rightarrow \text{lysophosphatidic acid} + \text{ADP}$$

has been found in soluble fractions from pig brain (35,36) and rat liver (37). In all these cases, the activity was closely associated with a diacylglycerol kinase activity. A lysophosphatidic acid phosphatase activity has been found in microsomes from avocado mesocarp, where monoacylglycerols were a major product of incubation with ^{14}C-glycerol or ^{14}C-glycerol-3-phosphate (27). It may, therefore, be a reversible monoacylglycerol/diacylglycerol kinase/phosphatase activity that is responsible for the accumulation of mono- and diacylglycerols in soluble fractions of developing rapeseed.

While microsomes and oil bodies from developing rapeseed rapidly incorporated oleoyl moieties from oleoyl-CoA into phosphatidic acids and phosphatidylcholines, relatively little triacylglycerol was formed as compared to microsomes from sunflower (7) or safflower (38). In other cases, however, the extent of incorporation of oleoyl moieties into triacylglycerols was close to that found in the present study, e.g., in safflower (21), sunflower (24), linseed (8) and mustard (12). It has been reported that, in the absence of glycerol-3-phosphate, phosphatidylcholine is mainly labeled by direct transfer of oleoyl moieties from oleoyl-CoA to lysophosphatidylcholine (10,22,24,29). In the presence of unlabeled glycerol-3-phosphate and a working Kennedy pathway, the equilibrium between phosphatidylcholines and diacylglycerols will result in the efflux of label from phosphatidylcholines and toward triacylglycerols (7,10).

In an attempt to stimulate the flow of oleoyl moieties from phosphatidylcholines toward triacylglycerols, a pulse-chase experiment was performed. Microsomal and oil body fractions of rapeseed were preincubated for 30 min with 20 nmol [1-^{14}C]oleoyl-CoA in the presence of 100 nmol lysophosphatidylcholine (Table 2). This ensured that the vast majority of oleoyl moieties incorporated were transferred to phosphatidylcholines. A large excess of unlabeled oleoyl-CoA (100 nmol) plus 100 nmol each of lysophosphatidic acid and glycerol-3-phosphate was then added to each incubation mixture, which were incubated for up to another two hours. Despite the large dilution of [1-^{14}C]oleoyl-CoA at 30 min, there was a further substantial incorporation of ^{14}C-oleoyl moieties upon the addition of lysophosphatidic acid and glycerol-3-phosphate, as shown in Table 2. No evidence of an efflux of ^{14}C-oleoyl moieties from phosphatidylcholines was observed, even after a further 2-hr incubation under conditions of active phosphatidic acid synthesis. The results from microsomal and oil body fractions were essentially similar. Oil bodies incorporated much more of the oleoyl moieties into phosphatidic acids in the absence of lysophosphatidic acid and responded to its presence with a 50% increase in this incorporation. Microsomes incorporated negligible proportions of oleoyl moieties into phosphatidic acids in the absence of lysophosphatidic acid (and the presence of lysophosphatidylcholine), but increased this incorporation 60-fold in the presence of lysophosphatidic acid. In both subcellular fractions, however, there was no increase in the labeling of any of the neutral lipids and no decrease in the labeling of phosphatidylcholines.

These results imply that the major activities present in both oil body and microsomal fractions of rapeseed

TABLE 2

Pulse Labeling of Lipids in the Subcellular Fractions of Developing Rapeseed with [1-^{14}C]Oleoyl-CoA Followed by Chase of Label upon the Addition of Glycerol-3-phosphate and Lysophosphatidic Acid

		Incorporation of oleoyl moieties into lipids (nmol·mg^{-1} protein)[a]				
Fraction	Time (min)	PC	PA	Other PL	TG	Other neutral lipids
Microsomes	30 + 0	26.0	0.4	0.3	0.4	0.7
	30 + 30	27.3	17.2	0.3	0.5	1.1
	30 + 60	26.4	24.3	0.2	0.6	1.2
	30 + 120	25.1	24.5	0.3	0.4	0.9
Oil bodies	30 + 0	18.6	9.1	4.0	2.3	3.1
	30 + 30	19.9	13.9	2.1	2.5	1.8
	30 + 60	19.9	14.0	2.0	2.6	2.9
	30 + 120	20.0	14.1	1.8	2.5	3.0

PC, phosphatidylcholines; PA, phosphatidic acids; PL, phospholipids; TG, triacylglycerols.
[a]Microsomes and oil bodies from developing rapeseed were incubated for 30 min with 3.7 KBq [1-^{14}C]oleoyl-CoA (20 nmol) in the presence of 100 nmol lysophosphatidylcholine. Thereafter, 100 nmol each of unlabeled oleoyl-CoA, glycerol-3-phosphate and lysophosphatidic acid were added and the incubations were continued for various periods. Incorporation of oleoyl moieties into various lipids was determined. Values are the means of at least two independent experiments.

were the lysophospholipid acyltransferases leading to the formation of phosphatidic acids and phosphatidylcholines. These activities were almost entirely absent from the soluble fraction. In the presence of appropriate acyl acceptors, these activities dwarfed the other acyltransferases and channeled as much as 95% of the oleoyl moieties from the added oleoyl-CoA onto a particular phospholipid. Although both fractions, and particularly the oil bodies, were capable of modest rates of triacylglycerol synthesis, the huge increase in the labeling of phosphatidic acids in the presence of exogenous lysophosphatidic acid did not lead to correspondingly large increases in triacylglycerol (or even diacylglycerol) formation (Table 2). This could possibly be explained by the relatively large endogenous diacylglycerol pool in the developing rapeseed fractions used in the present study. If the two reactions that are believed to channel acyl moieties into the diacylglycerol pool are truly equilibrium reactions, i.e.,

$$\text{phosphatidylcholine} \underset{\text{CMP}\ \ \text{CDP-choline}}{\overset{\text{CMP}\ \ \text{CDP-choline}}{\rightleftharpoons}} \text{diacylglycerol}$$

$$\text{phosphatidic acid} + \text{ADP} \longleftrightarrow \text{diacylglycerol} + \text{ATP}$$

then the presence of a large diacylglycerol pool would effectively prevent any large-scale flow of labeled oleoyl moieties toward triacylglycerols. The soluble fraction, which contained no detectable endogenous diacylglycerols, actively incorporated oleoyl moieties into diacylglycerols, but apparently lacked the further acyltransferase responsible for triacylglycerol formation. Such a diacylglycerol acyltransferase from a particulate fraction from safflower seeds has recently been described (38).

The effect on metabolism of oleoyl-CoA of incubating mixtures of the various subcellular fractions is shown in Table 3. It is clear that in each case the various fractions exhibited their characteristic patterns of metabolism of oleoyl-CoA with the result that an average labeling pattern was obtained. For example, when the microsomal and soluble fractions were incubated together, the labeling pattern was approximately the same as if an average had been taken of each fraction's individual labeling pattern. The total incorporation of ^{14}C-oleoyl moieties was greatly reduced in the combined fractions compared to the sum of the individual fractions.

In one experiment, the oil bodies from rapeseed were delipidated by three gentle extractions with pentane. This led to removal of most of the storage oil without denaturing the proteins associated with the oil bodies, since the oleate incorporation activities were similar both before and after the delipidation (Table 3). Therefore, the lipid-synthesizing enzymes present in the oil body fraction of rapeseed can function in the absence of most of the oil.

To summarize, it was found that microsomal, oil body and soluble fractions of developing rapeseed were all capable of high rates of metabolism of oleoyl-CoA. The microsomal and oil body fractions possessed all the activities of the Kennedy pathway for triacylglycerol biosynthesis. The major activities in these fractions were the lysophospholipid acyltransferases responsible for the formation of phosphatidic acids and phosphatidylcholines. At present it is not clear whether these are separate enzymes, but the lack of acyltransferase in the presence of lysophosphatidylethanolamine and lysophosphatidylinositol rules out a single, completely nonspecific lysophospholipid acyltransferase able to utilize any lysophospholipid substrate. The flow of acyl groups further along the Kennedy pathway toward triacylglycerols may have been inhibited by the presence of a large endogenous

TABLE 3

Incorporation of Oleoyl Moieties into Lipids of Subcellular Fractions of Developing Rapeseed and Their Mixtures upon Incubation with [1-^{14}C]Oleoyl-CoA

Fraction	Incorporation of oleoyl moieties into lipids (nmol·mg^{-1} protein)[a]					
	MG	DG	TG	PA	PC	Other PL
Microsomes	2.0	2.3	1.0	1.2	5.6	0.6
Oil bodies	2.0	1.9	1.4	2.7	5.5	2.7
Soluble fraction	5.2	10.3	tr	0.9	0.3	0.1
Microsomes + soluble fraction	2.5	3.0	0.3	0.4	3.0	0.3
Oil bodies + soluble fraction	2.1	1.8	0.7	1.8	2.3	1.6
Microsomes + oil bodies + soluble fraction	3.0	3.2	0.2	0.7	2.0	0.6
Oil bodies (delipidated)	1.4	2.2	1.6	1.0	6.8	1.9

MG, monoacylglycerols; DG, diacylglycerols; TG, triacylglycerols; PA, phosphatidic acids; PC, phosphatidylcholines; PL, phospholipids; tr, traces.

[a]Microsomes, oil bodies, delipidated oil bodies and soluble fraction from developing rapeseed and their mixtures were incubated with 3.7 KBq [1-^{14}C]oleoyl-CoA (20 nmol), and the incorporation of oleoyl moieties into various lipids was determined. Values are the means of at least two independent experiments.

diacylglycerol pool in these fractions, i.e., by keeping the equilibrium between phosphatidic acids and diacylglycerols very much toward the former. In contrast, the soluble fraction, which had no endogenous diacylglycerols, mainly formed mono- and diacylglycerols. This fraction was, however, unable to form triacylglycerols or phosphatidylcholines in any significant quantity, but was capable of some phosphatidic acid synthesis—although, unlike the other fractions, the latter activity was not stimulated in the presence of lysophosphatidic acid. The apparent presence of glycerol-3-phosphate acyltransferase activity in all three subcellular fractions of rapeseed is of interest considering recent reports on the properties of this enzyme in both microsomes of safflower seed (26) and a soluble fraction from cocoa seed (16).

It is also clear from the present study that the enzymes responsible for the biosynthesis of triacylglycerols are by no means exclusively or even mostly localized in the microsomal fraction, as has been often assumed hitherto.

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The Kinetics of the Autoxidation of Polyunsaturated Fatty Acids

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The kinetics of the autoxidation of a series of polyunsaturated fatty acids (PUFA) with increasing degrees of unsaturation and the mono-, di- and triglycerides of linoleate have been studied in homogeneous chlorobenzene solution at 37 C under 760 torr of oxygen. The autoxidations were initiated by thermal decomposition of azo initiators and followed by measuring the rate of oxygen uptake. The rate of chain initiation was determined by the induction period method using α-tocopherol as the chain-breaking antioxidant. The measured oxidizabilities of the PUFA are linearly dependent on the number of doubly allylic positions present in the molecule. Thus, the oxidizability of linoleate is 2.03×10^{-2} $M^{-1/2}$ $sec^{-1/2}$, and the value for docosahexaenoate is five times greater, 10.15×10^{-2} $M^{-1/2}$ $sec^{-1/2}$. The rate of autoxidation for all PUFA studied and for the mono- and diglyceride is proportional to the substrate concentration and to the square root of the rate of chain initiation, implying that the autoxidation of these compounds follows the usual kinetic rate law. The autoxidation of the triglyceride is more complex and does not appear to follow the same rate law at all substrate concentrations. This deviation from the usual kinetic rate expression may be due to lipid aggregation at low concentrations of the triglyceride.
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The autoxidation of polyunsaturated fatty acids (PUFA) has received much attention due to its involvement in food spoilage and the relevance of lipid peroxidation in vivo to membrane damage, aging, heart disease and cancer (1-3). Many workers have studied the quantitative aspects of autoxidation to better understand the kinetics and mechanisms of these important reactions (4-6). However, these workers have concentrated on linoleate (18:2) and linolenate (18:3). The previous very early work (7) on the relative rate of oxygen uptake by the more highly unsaturated PUFA did not control the rates of radical initiation. Therefore, we have reinvestigated this important area of current interest.

This paper presents the first quantitative kinetics study of the more highly unsaturated PUFA, arachidonate (20:4) and docosahexaenoate (22:6) as well as comparative data on 18:2 and 18:3 and the mono-, di- and triglycerides of 18:2. These results should be useful in the quantitative evaluation of the autoxidation of mixtures of PUFA in biological systems.

The autoxidation of many organic materials is known to be a free radical chain process which, in homogeneous solution at sufficient oxygen pressures, proceeds by the mechanism shown (Scheme 1) (8,9). RH represents the organic substrate, in this case a PUFA molecule, and R· is the carbon-centered radical formed by abstraction of a doubly allylic hydrogen atom. The carbon-centered radical, R·, rapidly reacts with molecular oxygen to give the chain-carrying peroxyl radical, ROO· [2] (10). In the steady-state analysis of this reaction scheme, the rate of oxygen consumption is given by equation 5, where k_p and

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INITIATION:

$$RH \rightarrow R\cdot \qquad \text{RATE} = R_i \qquad [1]$$

PROPAGATION:

$$R\cdot + O_2 \xrightarrow{k_o} ROO\cdot \qquad [2]$$

$$ROO\cdot + RH \xrightarrow{k_p} ROOH + R\cdot \qquad [3]$$

TERMINATION:

$$2ROO\cdot \xrightarrow{2k_t} \text{NONRADICAL PRODUCTS} + O_2 \qquad [4]$$

SCHEME 1

k_t are the rate constants for the rate-controlling propagation step [3] and chain termination step [4], respectively. The ratio of these rate constants, $k_p/(2k_t)^{1/2}$, is referred to as oxidizability and is a measure of the ease with which the substrate will undergo autoxidation.

$$-d[O_2]/dt = \{k_p/(2k_t)^{1/2}\}[RH]R_i^{1/2} \qquad [5]$$

To allow quantitative studies on the kinetics of autoxidation, the rate of chain initiation, R_i, must be controlled and measurable. This is generally achieved by using thermally labile azo initiators, which decompose to give two radicals at a known and constant rate, as shown in equation 6, where I· is any organic molecular fragment.

$$I-N=N-I \xrightarrow{k_d} 2I\cdot + N_2 \qquad [6]$$

$$I\cdot + O_2 \xrightarrow{RH} IOOH + R\cdot \qquad [7]$$

The rate of initiation, R_i, is governed by the rate constant for the rate of decomposition of the initiator, k_d, as well as by the efficiency of the primordial radicals, I·, initiating equation 7 rather than recombining in the solvent cage in which they are formed. The value of R_i is generally measured by the induction period method (11), using equation 8, where ArOH is a phenolic antioxidant.

$$R_i = n[ArOH]/\tau \qquad [8]$$

Determination of the time, τ, that the rate of oxygen uptake is inhibited allows the calculation of R_i if n, the stoichiometric factor, is known. (The factor n is defined as the number of radicals trapped by each molecule of antioxidant.) α-Tocopherol is known to have an n value of 2 and is also one of the best phenolic antioxidants known (12). Once R_i is known, the initiator efficiency, e—the number of initiator radicals that initiate—can be calculated using equation 9. The kinetic chain length, KCL, is the number of substrate molecules oxidized per initiating radical, and can be obtained from equation 10.

$$e = R_i/2k_d[In] \qquad [9]$$

$$KCL = \{-d[O_2]/dt\}/R_i \qquad [10]$$

Values of $k_p/(2k_t)^{1/2}$ and the KCL for the autoxidation of PUFA can readily be obtained using the above kinetic expressions.

MATERIALS AND METHODS

Materials. Methyl linoleate (18:2) and ethyl linolenate (18:3), 99%, were obtained from Sigma Chemical Co. (St. Louis, Missouri). Methyl arachidonate (20:4), methyl docosahexaenoate (22:6) and the mono-, di- and triglycerides of 18:2, all greater than 99% pure, were obtained from Nu-Chek-Prep (Elysian, Minnesota). The diglyceride was a mixture of the 1,2- and 1,3-isomers. 2,2′-Azobis(2-methylpropionitrile) (AIBN) was obtained from Kodak Chemical (Rochester, New York). 2,2′-Azobis(2,4-dimethylvaleronitrile) (DMVN) was obtained from Polysciences (Warrington, Pennsylvania). Di-tert-butyl hyponitrite (DBHN) was prepared by published procedure (13). Chlorobenzene, HPLC grade, was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and stored over molecular sieves. D-α-Tocopherol (α-T) was obtained from Henkel Corp. (Minneapolis, Minnesota). All chemicals were used as received.

Autoxidation procedures. All oxidations were carried out at 37 C under 760 torr of O_2 in an automatic recording gas absorption apparatus similar to those described elsewhere (14,15). The apparatus consists of a calibrated Validyne DP 15-30 pressure transducer (±1.25 psi) and CD-12 transducer indicator connected to a strip chart recorder. Both the transducer and reaction vessel were thermostatted at 37 C (±0.05 C).

In a typical experiment, a known volume of a chlorobenzene solution of the appropriate PUFA was put in the reaction vessel. The vessel and solution were saturated with oxygen by passing a stream of the gas over the rapidly stirred solution for 15 to 20 min. After sufficient time was allowed for the solution to come to thermal equilibrium (10–15 min), a known volume of initiator in chlorobenzene was added using a long-needle microliter syringe. Oxygen uptake by the autoxidizing PUFA began immediately upon addition of the initiator. Rates of oxygen uptake by the solution were calculated from the slope of the linear recorder trace after correcting for nitrogen evolution and oxygen uptake by the initiator radicals and oxygen evolved by the termination reaction (4).

The rate of chain initiation, R_i, was determined by the induction period method (11). Known amounts of a chlorobenzene solution of α-tocopherol were added to the autoxidizing PUFA. Using n = 2 for α-T (12) and the length of time, τ, that the oxygen uptake is inhibited, R_i was calculated from equation 8, by plotting 2[α-T] against τ and calculating the slope. The oxidizability, $k_p/(2k_t)^{1/2}$, of the PUFA was determined by measuring the rate of oxygen uptake in several runs where the rate of initiation and the PUFA concentration had both been varied. From equation 5, the oxidizability is equal to the slope of a line obtained by plotting the rate of oxygen uptake against $[PUFA]R_i^{1/2}$. The reproducibility of this method is quite good, 1 or 2% based on the errors in the slopes, and the absolute oxidizability values reported here are estimated to be accurate to ±10%.

The rate constants for the decomposition of the initiators, k_d, were determined by measuring the rate of nitrogen evolution from a chlorobenzene solution of the initiator in the same apparatus used for the autoxidations (16). The initiator solutions were deoxygenated by bubbling with nitrogen in the apparatus for 15 to 20 min prior to the start of the experiment.

RESULTS

A typical trace representing the oxygen uptake of an autoxidizing solution of PUFA is shown in Figure 1 for 20:4, and includes both the uninhibited and the α-tocopherol–inhibited reactions. It should be noted that the rate of oxygen uptake is the same before and after the inhibition period. The length of the induction period, τ, together with the concentration of α-T, can be used to calculate the rate of chain initiation, R_i, from equation 8. A number of injections of α-T were made, with care taken that the rate returned to the uninhibited value before the next injection of α-T was made. By measuring τ for various concentrations of α-T, a plot was constructed based on equation 8. The slope of the resulting straight line is equal to R_i for that particular kinetic run, as shown in Figure 2.

The data shown in Table 1 for the various PUFA were obtained by varying the concentrations of both the PUFA and the initiator and determining R_i as described above. Then, if equation 5 is obeyed, a plot of the rate of oxygen uptake vs $[PUFA]R_i^{1/2}$ should give a straight line with the slope equal to the oxidizability for that particular PUFA. Figure 3 shows the data in Table 1 plotted for all PUFA studied. The oxidizabilities (in units of $M^{-1/2}$ $sec^{-1/2}$) obtained from these lines are 18:2, 2.03×10^{-2}; 18:3, 4.07×10^{-2}; 20:4, 5.75×10^{-2}; and 22:6, 10.15×10^{-2}.

The average e for AIBN in all the runs in Table 1 was 66 ± 7%. This is in good agreement with data on this initiator in other systems (17,18). The efficiency of DMVN was higher but also more variable, with an average of 78 ± 15%. It should be noted, however, that the oxidizability of the PUFA is independent of the initiator employed.

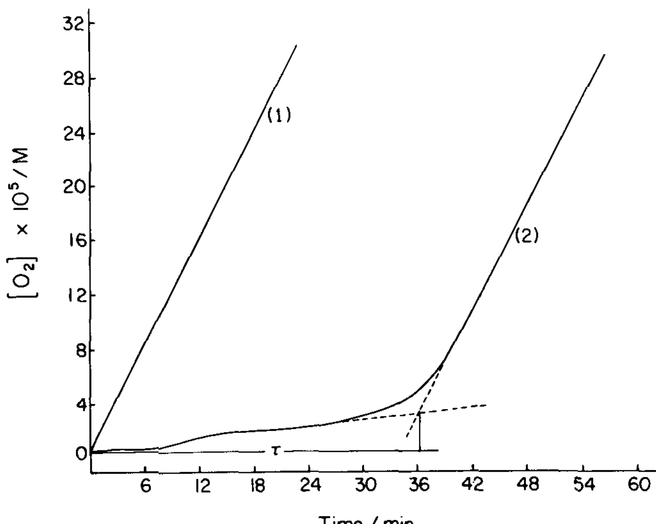

FIG. 1. The uninhibited (curve 1) autoxidation of 46.5 mM 20:4 in chlorobenzene initiated with 5.70 mM 2,2′-azobis(2,4-dimethylvaleronitrile) at 37 C. Curve 2 is the same reaction with 1.71×10^{-5} M α-tocopherol added.

TABLE 1

Autoxidation of PUFA at 37 C under 760 torr O_2

	Initiator (mM)	PUFA (M)	10^8 R_i (M sec^{-1})	$-10^6 d[O_2]/dt$ (M sec^{-1})[a]	$10^2 k_p/(2k_t)^{1/2}$ ($M^{-1/2}$ $sec^{-1/2}$)	KCL
18:2	24.5[b]	.0545	0.936	0.106	2.01	11
	4.05[c]	.2099	1.99	0.600	2.03	30
	62.1[b]	.6469	2.07	1.82	1.95	88
	63.9[b]	.8985	2.29	2.66	1.96	117
	8.90[c]	1.038	3.43	3.88	2.02	113
	4.49[c]	1.867	1.65	4.90	2.04	297
18:3	19.6[b]	.0934	0.856	0.336	3.89	39
	43.7[b]	.1868	2.02	1.05	3.96	52
	49.6[b]	.3735	1.95	2.26	4.33	116
	3.69[c]	.0815	1.40	3.91	4.12	279
	119.0[b]	.747	4.47	6.65	4.21	149
	205.0[b]	.747	7.55	8.24	4.02	109
20:4	5.70[c]	.0465	1.41	0.297	5.38	21
	56.0[b]	.0830	2.36	0.663	5.18	28
	49.9[b]	.166	2.00	1.32	5.62	66
	49.3[b]	.332	1.86	2.53	5.58	136
	107.0[b]	.332	4.38	3.93	5.65	90
	80.9[c]	.332	31.7	10.7	5.72	34
22:6	17.7[b]	.0525	0.881	0.504	10.23	58
	24.4[b]	.0867	1.12	0.934	10.17	83
	93.2[b]	.145	3.56	2.78	10.18	78
	56.0[b]	.260	2.31	3.88	9.82	168
	95.6[b]	.289	3.72	5.72	10.27	154

PUFA, polyunsaturated fatty acid; KCL, kinetic chain length.

[a]Rate corrected for N_2 given off and O_2 taken up by initiator and O_2 given off by termination reaction.

[b]2,2′-Azobis(2-methylpropionityrile); $k_d = 3.05 \times 10^{-7}$ sec^{-1}.

[c]2,2′-Azobis(2,4-dimethylvaleronitrile); $k_d = 2.43 \times 10^{-6}$ sec^{-1}.

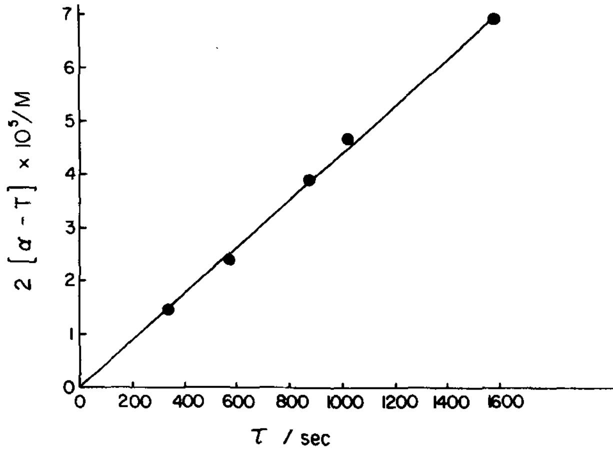

FIG. 2. Plot of $2[\alpha\text{-}T]$ vs τ for the autoxidation of 0.332 M 20:4 in chlorobenzene initiated with .1072 M 2,2-azobis(2-methylpropionitrile). Slope of the line equals R_i.

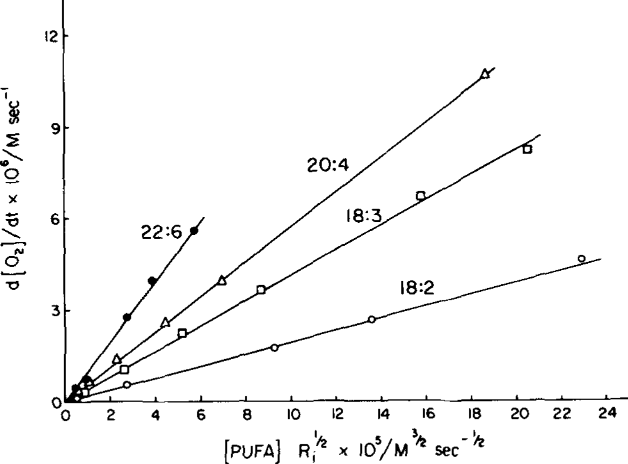

FIG. 3. Plots of $d[O_2]/dt$ vs $[PUFA]R_i^{1/2}$ for the four polyunsaturated fatty acids (PUFA) studied. Data taken from Table 1. ○, 18:2; □, 18:3; △, 20:4; ●, 22:6. The slope of the line equals the oxidizability of that PUFA.

The kinetic chain lengths (KCL) in these reactions were long, ranging from 10 to about 300. The long chain lengths together with the high efficiencies cause the correction due to nitrogen evolution and oxygen uptake by the initiators to be quite small, generally less than 5% of the measured rate of oxygen uptake.

To investigate the effects of more than one unsaturated fatty acid chain in the same lipid molecule, the oxidizabilities of the mono-, di- and triglycerides of 18:2 were measured. These determinations were done in the same manner as described for the individual PUFA; the results

TABLE 2

Autoxidation of Linoleoyl Glycerides at 37 C under 760 torr of O_2

	Initiator (mM)	PUFA (mM)	10^8 R_i (M sec $^{-1}$)	$-10^7d[O_2]/dt$ (M sec^{-1})[a]	$10^2k_p/(2k_t)^{1/2}$ ($M^{-1/2}$ × $sec^{-1/2}$)	KCL
Monoglyceride	1.21[b]	18.5	1.31	0.588	2.77	5
	5.95[c]	29.1	2.04	1.12	2.70	6
	12.7[c]	58.3	4.84	4.05	3.16	8
	10.1[c]	146	3.68	7.42	2.66	20
	7.69[b]	118	8.99	9.40	2.66	11
	7.24[c]	291	1.90	11.0	2.74	58
	7.69[b]	235	8.89	19.0	2.71	21
Diglyceride	1.23[c]	9.75	0.534	0.441	6.19	9
	2.29[c]	9.27	1.07	0.575	6.00	5
	1.33[c]	18.5	0.586	0.782	5.51	13
	2.42[c]	19.5	1.06	1.28	6.37	12
	2.23[b]	25.0	2.40	2.21	5.70	9
	10.1[b]	56.8	11.0	10.9	5.80	10
	38.6[c]	185.	14.5	40.0	5.89	17
Triglyceride	7.37[d]	5.07	0.302	0.441	15.8	15
	2.29[c]	6.23	1.09	0.863	13.3	8
	1.65[b]	4.79	2.46	1.17	15.6	5
	15.3[d]	10.7	0.615	1.25	14.9	20
	2.42[c]	13.1	1.17	1.69	11.9	14
	2.77[b]	10.1	3.13	2.75	15.4	9
	5.19[c]	23.5	2.53	2.84	7.59	11
	47.9[d]	32.1	2.05	4.22	9.17	21
	73.1[d]	48.2	3.04	6.854	8.15	23
	10.7[b]	58.0	5.25	10.0	7.52	19
	15.0[b]	43.6	17.4	18.8	10.3	11
	20.8[c]	87.1	10.2	21.6	7.77	21
	21.9[b]	111.	25.3	44.9	8.05	18

PUFA, polyunsaturated fatty acid; KCL, kinetic chain length.

[a]Rate corrected for N_2 given off and O_2 taken up by initiator and O_2 given off by termination reaction.

[b]Di-tert-butyl hyponitrile; $k_d = 8.57 \times 10^{-6}$ sec^{-1}, forms butoxyl radical upon decomposition, which does not react with oxygen.

[c]2,2′-Azobis(2,4-dimethylvaleronitrile); $k_d = 2.43 \times 10^{-6}$ sec^{-1}.

[d]2,2′-Azobis(2-methylpropionitrile); $k_d = 3.05 \times 10^{-7}$ sec^{-1}.

are shown in Table 2. The data for the monoglyceride of 18:2 give an oxidizability of 2.68×10^{-2} $M^{-1/2}$ $sec^{-1/2}$. This value is somewhat higher than that obtained for the methyl ester of 18:2. The average efficiencies for DMVN and DBHN in this system were 73 ± 13% and 66 ± 3%, respectively. The value for DMVN is similar to the efficiency reported for the individual PUFA, while the efficiency for DBHN was the same as that reported for other systems (4).

The diglyceride of 18:2 had a greater oxidizability than the monoglyceride, the value being 5.89×10^{-2} $M^{-1/2}$ $sec^{-1/2}$. While the efficiency with which DBHN initiated radical chains was about the expected value, 63% (4), the efficiency of DMVN was 88 ± 9%. This value is greater than the efficiency found with the other substrates studied.

The data on the triglyceride of 18:2, shown in Table 2, appear somewhat more complex. The least squares line of the plot of $d[O_2]/dt$ vs $[PUFA]R_i^{1/2}$ gives the oxidizability for the triglyceride as 7.98×10^{-2} $M^{-1/2}$ $sec^{-1/2}$. This value is three times that determined for the monoglyceride of 18:2. However, the oxidizability is not constant as the concentration of the ester is increased, as it is with the other substrates studied. Both AIBN and DBHN had about the expected efficiency when used with the triglyceride: 68 ± 2% and 72 ± 10%, respectively. However, the DMVN gave an average efficiency of 100 ± 2% with this substrate.

DISCUSSION

The autoxidation of many organic substances is a free radical chain process that obeys the kinetic expression in equation 5 (8,9). By control of the variables in that expression, it is possible to obtain a quantitative measure of the susceptibility of a substrate to undergo autoxidation, $k_p/(2k_t)^{1/2}$. Previous work on the rates of oxidation of PUFA reported that the relative maximum rates of autoxidation at 37 C were in the ratios 1:2:4:—:8 as the number of doubly allylic positions increased from 1 to 5 (7,19). However, that work was done by measuring the rate of oxygen uptake by spontaneously autoxidizing samples of pure PUFA, and no attempt was made to control or even measure the R_i. Using thermally labile azo initiators to control R_i and the induction period

method to accurately measure R_i, we have determined the oxidizabilities of a number of PUFA.

The linear correlations, shown in Figure 3, and the constancy of the data given in Table 1 imply that all PUFA obey the kinetic expression given by equation 5. We determined the values of $k_p/(2k_t)^{1/2}$ for 18:2 and 18:3 to be 2.03×10^{-2} $M^{-1/2}$ $sec^{-1/2}$ and 4.07×10^{-2} $M^{-1/2}$ $sec^{-1/2}$ at 37 C; these values compare quite well with the values of 2.1×10^{-2} $M^{-1/2}$ $sec^{-1/2}$ and 3.9×10^{-2} $M^{-1/2}$ $sec^{-1/2}$ at 30 C measured by Howard and Ingold (20). Our results on the more highly unsaturated PUFA, 20:4 and 22:6, are 5.75×10^{-2} and 10.15×10^{-2} $M^{-1/2}$ $sec^{-1/2}$, respectively, and are among the highest substrate oxidizabilities reported at 37 C, demonstrating the susceptibility of highly unsaturated compounds to autoxidation.

Our data for the oxidizability of PUFA indicate that these values are linearly related to the number of doubly allylic positions present in the molecule. As the number of doubly allylic positions increases in the series from 1:2:3:—:5, the ratio of the oxidizabilities increases to 1:2:3:—:5. At equal concentrations of PUFA and equal rates of initiation, the rate of oxygen uptake follows this same ratio. The previously reported ratio of oxidizabilities for the PUFA series from 18:2 to 22:6 of 1:2:4:—:8, therefore, is not correct.

The linear relationship between ease of autoxidation and the number of doubly allylic positions predicts an oxidizability of about 2.0×10^{-2} $M^{-1/2}$ $sec^{-1/2}$ per activated methylene group at 37 C. This relationship allows us to speculate on the oxidizability of docosapentaenoate (22:5), which we did not study. (This compound is not readily available commercially in sufficient quantity and purity to allow accurate kinetic studies.) Based on an oxidizability of 2.0×10^{-2} $M^{-1/2}$ $sec^{-1/2}$ per doubly allylic position, 22:5 with four active methylene groups, should have an oxidizability of about 8.0×10^{-2} $M^{-1/2}$ $sec^{-1/2}$. The large oxidizability values for the highly unsaturated PUFA, 22:5 and 22:6, can quantitatively explain why tissues rich in these substances, such as the retina, are so susceptible to free radical damage (21,22).

The use of the mono-, di- and triglycerides of 18:2 allows us to investigate the effect of more than one unsaturated chain contained in the same molecule. The oxidizability for the monoglyceride of 18:2 was determined to be 2.68×10^{-2} $M^{-1/2}$ $sec^{-1/2}$. As with the other PUFA esters, the measured oxidizability for the monoglyceride was linearly dependent on the substrate concentration and on the square root of the rate of initiation and also independent of the type of initiator used. With the PUFA and the monoglyceride, DMVN was the most efficient initiator, with an average of about 75 ± 14%, similar to that reported in previous work in homogeneous solution (23). The DBHN and AIBN behaved as expected with all the PUFA and the monoglyceride. The average efficiencies are in agreement with those reported in the literature for other systems: 66 ± 7% for AIBN (17,18) and 66 ± 3% for DBHN (4).

The difference in oxidizability between the monoglyceride and the methyl ester of 18:2, although small, is of interest. The oxidizability should be dependent on the number of activated methylene groups in the fatty acid carbon chain and should be independent of the ester functionality, and the methyl ester and the monoglyceride would be expected to have the same oxidizability. The small difference between the oxidizability of 18:2 and the monoglyceride is possibly due to the more polar nature of the latter. The chain-carrying peroxyl radicals have some polar character in the ground state that is lost in the termination reaction (24,25). The polar nature of the ester functionality in the monoglyceride may serve to inhibit the termination reaction slightly, decreasing $2k_t$ and resulting in a larger value of $k_p/(2k_t)^{1/2}$ for the monoglyceride.

On the basis of doubly allylic positions per molecule, the oxidizability of the diglyceride should be twice that of the monoglyceride. However, for a molecule with two separate oxidizable side chains held close to one another in space, k_p could be increased relative to two individual molecules. This would imply that the diglyceride would have a larger oxidizability than predicted on the basis of the number of doubly allylic positions present in the molecule. We determined the oxidizability of the diglyceride to be 5.89×10^{-2} $M^{-1/2}$ $sec^{-1/2}$ and found that this compound also obeyed the kinetics represented by equation 5 in the concentration ranges studied. This value is more than twice the value found for the monoglyceride, but when one considers the errors involved in these measurements, the significance of the difference is questionable. However, the diglyceride was a mixture of the 1,2- and 1,3-isomers of dilinoleoyl glycerol. To fully investigate the effect of the relative positions of the two linoleate chains in a diglyceride, it would be interesting to determine the oxidizability of the pure 1,2- and 1,3-isomers.

The determination of the oxidizability of the triglyceride of 18:2 was of interest because this compound has three linoleate chains held in close proximity to one another. However, the kinetics of the autoxidation of the triglyceride seem more complex than for the other substrates studied. The plot of $d[O_2]/dt$ vs $[PUFA]R_i^{1/2}$ gives a slope of 7.98×10^{-2} $M^{-1/2}$ $sec^{-1/2}$, exactly three times the value found for the monoglyceride. However, based on the present data, the triglyceride does not appear to follow classical autoxidation kinetics. The oxidizability decreases by about a factor of 2 as the concentration of triglyceride is increased at constant R_i (i.e., see lines 3 and 7 for triglyceride in Table 2). For a compound that obeys equation 5, the overall order of the reaction with respect to $[PUFA]R_i^{1/2}$ should be 1.0. In fact, for all substrates studied, except the triglyceride, the average overall order is 1.002 ± .014, in excellent agreement with the theoretical value. However, for the triglyceride, the overall order is about 0.84. While the absolute value of this number is not significant, the fact that it is not 1.0 indicates that the triglyceride does not obey the kinetic expression given in equation 5. This deviation from the standard kinetics appears greatest at low triglyceride concentrations. The data show that as the concentration of triglyceride increases, the overall order of the reaction with respect to $[PUFA]R_i^{1/2}$ approaches 1.0. This change in reaction order may be due to lipid aggregation at low concentrations of the triglyceride. It has been shown that phospholipids in homogeneous organic solution do not follow the classical autoxidation kinetics, reportedly due to lipid aggregation (26). While aggregation may be occurring with the triglyceride, it seems likely that the mono- and diglyceride would also form aggregates. However, aggregate formation may occur at different concentrations for the three glycerides and a wider range of

substrate concentrations needs to be studied to probe this point. Since equation 5 is not followed for the triglyceride of 18:2, the reported oxidizability is of little significance, and this substance will require a more thorough investigation.

The triglyceride also appears to give anomalous e values for one of the initiators. Using PUFA and the monoglyceride as substrates, AIBN and DBHN gave e equal to about 66% and DMVN about 75%, values expected from previous work. Both DBHN and AIBN were well behaved with the di- and triglyceride, giving the same e value as reported above. For DMVN, however, e increased to 88 ± 9% for the diglyceride and to 100 ± 2% for the triglyceride. We are not able to explain these increases in e, but they are not due to an increase in k_d for DMVN in the presence of the di- or triglyceride. This implies that these two glycerides somehow alter the solvent cage around the initiator molecules, allowing more radicals to escape the cage. Why this would occur only for DMVN and not DBHN or AIBN is not clear.

In conclusion, we have shown that the oxidizability of simple PUFA esters is directly related to the number of doubly allylic positions present in the molecule. This relationship does not appear to apply to the mono-, di- and triglycerides of 18:2, however. In fact, the triglyceride does not follow the classical autoxidation kinetics, and more work is required to elucidate the kinetics and mechanisms of its autoxidation.

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Net Lipid Transfer Between Lipoproteins in Fish-Eye Disease Plasma Supplemented with Normal High Density Lipoproteins

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Native fish-eye disease plasma, which is deficient of both high density lipoproteins (HDL) and lecithin–cholesterol acyltransferase activity (α-LCAT), processing the free cholesterol of these lipoproteins, has been supplemented with normal isolated HDL_2 or HDL_3 and incubated in vitro at 37 C. After incubation for 0, 7.5 and 24 hr the very low density (VLDL) and low density (LDL) lipoproteins as well as HDL were isolated, and their contents of triglycerides, phospholipids and free, esterified and total cholesterol were quantified. The resulting net mass transfer of the different lipids revealed a functioning transfer of cholesteryl esters and all other analyzed lipids between the lipoproteins, although no de novo esterification of the HDL cholesterol by LCAT in this plasma occurred. In accordance with previous findings there was a functioning esterification process of the free cholesterol of the combined VLDL and LDL of fish-eye disease plasma. The present results make it reasonable to conclude that the lack of HDL cholesterol esterification in this disease is not a result of a deficiency of cholesteryl ester transfer or lipid transfer activities.
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Fish-eye disease (FED) is a familial dyslipoproteinemia (1,2), characterized by hypoalphalipoproteinemia associated with a specific deficiency of high density lipoprotein lecithin–cholesterol acyltransferase activity (α-LCAT) in plasma (3), but having a normally functioning cholesterol esterification activity acting on the combined very low density (VLDL) and low density (LDL) lipoproteins (β-LCAT) (3,4). Of several possible defects that might cause deficiency of α-LCAT, one would be a defective or deficient lipid transfer protein acting together with a postulated complex composed of LCAT and apolipoproteins forming a "cholesteryl ester transfer complex" (5) that could both synthesize and distribute cholesteryl esters to acceptor lipoproteins.

It has previously been demonstrated (6) that gross lipid transfer protein activities were normal when estimated in lipoprotein-depleted FED plasma. However, in light of the recently formulated hypothesis of α- and β-LCAT activities in plasma (3), the deficiency of α-LCAT activity in FED might be associated with or caused by a malfunctioning lipid transfer protein specific for high density lipoprotein (HDL) cholesteryl esters.

Determination of the flux of the cholesteryl esters of FED HDL in whole plasma is, however, hampered by the fact that the plasma cholesterol concentration level and the cholesteryl ester percentage of this lipoprotein are reduced by 90% and 75%, respectively, compared to normal plasma (7).

In the present investigation we have studied the in vitro incubation-induced changes of the lipid compositions of VLDL, LDL and HDL of FED plasma supplemented with normal HDL subfractions, with and without LCAT inhibitor, in order to evaluate the quality of the cholesteryl ester transfer activity in such plasma. The results are reported herein.

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EXPERIMENTAL PROCEDURES

The FED patient no. 1 (S.R.) has been described in detail previously (1,2). Venous blood was taken from the patient in the mornings after overnight fast into vacutainer plastic tubes containing disodium EDTA. The tubes were immediately placed into crushed ice. Plasma was recovered within 30 min by low speed centrifugation at 2 C and was stored in crushed ice. Merthiolate® normally added to plasma in our work with lipoproteins was omitted. Free cholesterol and cholesteryl esters were quantified enzymatically in duplicate, directly on aliquots of plasma and on the lipoprotein fractions obtained after ultracentrifugation, by means of Merck kits 14106, 14107 and 14108 (Darmstadt, Federal Republic of Germany). The lipids of the plasma lipoproteins VLDL, LDL and HDL were extracted with chloroform/methanol (v/v) (8). Phospholipids and triglyceride concentrations were estimated in triplicate and duplicate, respectively, on aliquots of the chloroform phase as previously described (9,10). The coefficients of variation (number of estimates in parentheses) were 2.7% (75), 3.5% (57), 3.1% (57) and 1.1% (36) for between-run reproducibility of determinations of triglycerides, free cholesterol, esterified cholesterol and phospholipids, respectively. Concentrated normal HDL_2 and HDL_3 were defined as HDL subfractions (11) with density regions between 1.070 and 1.125 kg/l and 1.125 and 1.210 kg/l, respectively, and were isolated by preparative ultracentrifugation and dialyzed against 0.15 mol/l sodium chloride as described previously (Holmquist, L., and Carlson, L.A., submitted for publication). As a test for possible contamination of isolated normal HDL preparations by LCAT activity, preparations were mixed with a solution of essentially fatty acid-free bovine serum albumin, 5% final concentration, and incubated for 24 hr at 0 and 37 C. Estimations of free cholesterol and cholesteryl ester contents of the incubation mixtures showed that less than 14% of the free cholesterol was converted to cholesteryl esters after 24 hr of incubation (96% was obtained on incubations with autologous lipoprotein-depleted plasma), and no change of particle size could be detected. This indicates that HDL-associated LCAT activity is negligible in the HDL preparations obtained by the present method of preparation (11). Polyacrylamide gradient gel electrophoresis was performed as previously reported (2). Isolations of plasma lipoproteins (VLDL [d < 1.006 kg/l], LDL [d = 1.006–1.063 kg/l] and HDL [d = 1.063–1.21 kg/l]) after the incubation experiments were made by preparative ultracentrifugation at 1 C on a routine basis (12) using a Beckman L8-55 ultracentrifuge equipped with a type 50.3 Ti rotor and Quick-Seal Ultra-Clear tubes (6 ml) (Beckman Instruments, Palo Alto, California).

Incubation experiments. Ice-cold FED plasma (11.0 and 9.50 ml for HDL_2 and HDL_3 experiments, respectively) obtained as described above was mixed with concentrated HDL_2 (0.70 ml) or HDL_3 (1.90 ml) preparations (11). In experiments with LCAT inhibitor, 0.12–0.14 ml of phosphate-buffered 100 mmol/l disodium 5,5′-dithiobis-(2-nitrobenzoate) (DTNB), pH 7.4, was added to the plasma mixtures to 1 mmol/l final concentration. By addition of 0.15 mol/l sodium chloride solution, the final volumes of the incubation mixtures were adjusted to 13.3 and 11.5 ml for experiments with HDL_2 and HDL_3, respectively, resulting in 17% dilution of the original plasma. In experiments with native FED plasma, 0.15 mol/l sodium chloride solution was added to give the same final dilution as above, thus maintaining the LCAT and transfer protein concentrations equal in all incubation experiments.

The final concentrations of free cholesterol originating from HDL_2 or HDL_3 in the incubation mixtures were equimolar and adjusted to yield values representative of their average abundance in normal plasma.

Table 1 shows the compositions and cholesterol concentrations for the different incubation mixtures. Incubations were performed at 37 C in the dark under N_2 atmosphere.

RESULTS

Table 2 shows the concentration values for triglycerides, total, free and esterified cholesterol, cholesteryl ester percentage and phospholipids in VLDL, LDL and HDL fractions, estimated at 0, 7.5 and 24 hr of incubation of mixtures of FED plasma, alone or supplemented with normal isolated HDL_2 or HDL_3. The corresponding values obtained at incubation experiments of the same designs as above but performed in the presence of the LCAT inhibitor DTNB, run in parallel, are shown in Table 3.

Triglycerides. The triglycerides of the VLDL fraction of the different incubation mixtures decreased by 0.4–0.5 mmol/l, independently if β-LCAT was active or inhibited by DTNB. There was a concomitant increase in LDL triglycerides corresponding to about 0.4 mmol/l in native FED plasma and in such plasma supplemented with HDL_2. The increase in LDL triglycerides in plasma supplemented with HDL_3 was somewhat lower, about 0.3 mmol/l; however, this was associated with a correspondingly higher increase in triglycerides, 0.1 mmol/l, of the HDL fraction of the HDL_3-supplemented plasma compared to the 0.06 mmol/l triglyceride increase in the HDL fraction in experiments with HDL_2. The triglyceride concentrations of HDL in native FED plasma were too low to estimate accurately. The observed increase in triglycerides of LDL and HDL_2 or HDL_3 were of the same order whether LCAT was active or inhibited. Thus the decrease in VLDL triglyceride concentrations in all incubation experiments was completely balanced by the sum of the increase in triglycerides of the matching LDL and HDL fractions.

Cholesteryl esters. In the presence of the LCAT inhibitor DTNB, the cholesteryl ester concentration of VLDL of all three incubation mixtures increased by about 0.4 mmol/l on incubation. This increase in VLDL cholesteryl esters of the native FED plasma reflected a decrease in cholesteryl ester content by about 0.3 mmol/l of the LDL fraction of this plasma. The decrease in cholesteryl ester concentration of FED HDL was not possible to estimate accurately as its concentration was too low.

The decrease in cholesteryl ester content of LDL in plasma supplemented with HDL_2 or HDL_3 was 0.06 and 0.08 mmol/l lower, respectively, than that for LDL in native FED plasma. This reduced decrease in cholesteryl esters of such LDL compared to native plasma LDL was matched, however, by a decrease in cholesteryl ester concentration of 0.12 and 0.23 mmol/l in HDL_2 and HDL_3, respectively, in corresponding incubation experiments with these lipoproteins. Thus the molar increase in cholesteryl esters of the VLDL fractions corresponded in all experiments to the sum of the decrease in these lipids of LDL and HDL lipoproteins. In none of these experiments with LCAT inhibitor was there any de novo synthesis of whole plasma cholesteryl esters in 24 hr of incubation.

In the corresponding incubation experiments without LCAT inhibitor, the cholesteryl ester content of VLDL increased by 0.3–0.4 mmol/l. In LDL of incubated native FED plasma, a rise (0.06 mmol/l) of cholesteryl esters could also be observed. In the HDL fraction of this plasma, which had an extremely low cholesteryl ester content, no change of this lipid could be observed. In the LDL fractions of the HDL_2- and HDL_3-supplemented plasmas there were 0.34 and 0.33 mmol/l increases of the cholesteryl ester content, respectively, while the cholesteryl ester content of the HDL_2 and HDL_3 fractions decreased by about 0.1 and 0.2 mmol/l, respectively.

In the LCAT-active incubation mixtures of native FED

TABLE 1

Origin and Final Concentrations (mmol/l) of Free and Esterified Cholesterol in Incubation Mixtures of Fish-Eye Disease Plasma and Isolated Normal HDL_2 or HDL_3 Fractions at Start of Incubation at 37 C

	Originating from plasma		From added HDL_2		From added HDL_3		Total in mixture of plasma and HDL_2 or HDL_3		
	FC	CE	FC	CE	FC	CE	FC	CE	TC
Plasma + NaCl	2.37	2.90	—	—	—	—	2.37	2.90	5.27
Plasma + HDL_2	2.37	2.90	0.12	0.26	—	—	2.49	3.16	5.63
Plasma + HDL_3	2.37	2.90	—	—	0.12	0.47	2.49	3.37	5.86

Same values for experiments with disodium 5,5′-dithiobis-(2-nitrobenzoate). HDL, high density lipoprotein; FC, free cholesterol; CE, esterified cholesterol; TC, sum of free and esterified cholesterol.

TABLE 2

Concentrations of Triglycerides (TG), Phospholipids (PL), Free (FC), Esterified (EC) and Total Cholesterol (TC) (mmol/l Incubation Mixture) and Cholesteryl Ester Percentage (%) of the VLDL, LDL and HDL Fractions of Native Fish-Eye Disease Plasma and Such Plasma Supplemented with Normal Isolated HDL_2 or HDL_3, Estimated at Different Times of Incubation at 37 C

Composition of mixture	VLDL						LDL						HDL					
	TG	PL	FC	CE	TC	CE (%)	TG	PL	FC	CE	TC	CE (%)	TG	PL	FC	CE	TC	CE (%)
Plasma + NaCl																		
0 hr	1.62	0.64	0.47	0.48	0.95	51	2.09	2.03	1.83	2.41	4.24	57	0.02	0.16	0.07	0.01	0.08	—
7.5 hr	1.32	0.57	0.45	0.68	1.13	60	2.36	1.97	1.59	2.46	4.05	61	0.03	0.07	0.02	0.01	0.02	—
24 hr	1.10	0.49	0.41	0.80	1.21	66	2.47	1.83	1.33	2.47	3.80	65	0.02	0.04	0.00	0.01	0.01	—
Plasma + HDL_2																		
0 hr	1.63	0.67	0.47	0.48	0.95	51	1.96	2.03	1.79	2.37	4.16	57	0.07	0.39	0.18	0.24	0.42	57
7.5 hr	1.31	0.58	0.47	0.71	1.18	60	2.21	1.98	1.60	2.51	4.11	61	0.13	0.32	0.10	0.16	0.26	62
24 hr	1.11	0.52	0.47	0.84	1.31	64	2.49	1.99	1.54	2.71	4.25	64	0.13	0.27	0.07	0.15	0.22	68
Plasma + HDL_3																		
0 hr	1.66	0.65	0.50	0.47	0.97	48	2.09	2.12	1.92	2.49	4.41	56	0.09	0.63	0.20	0.42	0.62	68
7.5 hr	1.31	0.52	0.51	0.69	1.20	58	2.23	1.98	1.82	2.62	4.44	59	0.21	0.61	0.15	0.29	0.44	66
24 hr	1.18	0.51	0.47	0.83	1.30	64	2.41	1.97	1.62	2.82	4.44	64	0.18	0.47	0.11	0.23	0.34	68

TC, Total cholesterol as sum of free and esterified cholesterol; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

TABLE 3

Concentrations of Triglycerides (TG), Phospholipids (PL), Free (FC), Esterified (CE) and Total Cholesterol (TC) (mmol/l Incubation Mixture) and Cholesteryl Ester Percentage (%) of the VLDL, LDL and HDL Fractions of Native Fish-Eye Disease Plasma and Such Plasma Supplemented with Normal Isolated HDL_2 or HDL_3, Estimated at Different Times of Incubation in the Presence of the LCAT Inhibitor DTNB at 37 C

Composition of mixture	VLDL						LDL						HDL					
	TG	PL	FC	CE	TC	CE (%)	TG	PL	FC	CE	TC	CE (%)	TG	PL	FC	CE	TC	CE (%)
Plasma + NaCl + DTNB																		
0 hr	1.68	0.64	0.50	0.47	0.97	48	2.06	2.05	1.91	2.42	4.33	56	0.04	0.21	0.10	0.01	0.11	—
7.5 hr	1.48	0.64	0.55	0.70	1.27	55	2.26	2.10	1.85	2.22	4.07	55	0.03	0.13	0.06	0.01	0.07	—
24 hr	1.28	0.59	0.56	0.83	1.39	60	2.49	2.22	1.84	2.11	3.95	53	0.03	0.09	0.04	0.01	0.05	—
Plasma + HDL_2 + DTNB																		
0 hr	1.70	0.65	0.50	0.48	0.98	49	2.02	2.10	1.93	2.48	4.41	56	0.07	0.40	0.19	0.24	0.43	56
7.5 hr	1.43	0.61	0.56	0.74	1.30	57	2.24	2.15	1.88	2.37	4.25	56	0.12	0.39	0.16	0.14	0.30	47
24 hr	1.31	0.64	0.62	0.90	1.52	59	2.42	2.18	1.85	2.23	4.08	55	0.12	0.36	0.14	0.12	0.26	46
Plasma + HDL_3 + DTNB																		
0 hr	1.66	0.65	0.50	0.48	0.98	49	2.02	2.08	1.89	2.46	4.35	57	0.10	0.65	0.19	0.42	0.65	69
7.5 hr	1.41	0.61	0.55	0.73	1.28	57	2.09	1.90	1.72	2.27	3.99	57	0.22	0.76	0.20	0.30	0.50	60
24 hr	1.27	0.62	0.59	0.88	1.47	60	2.35	2.02	1.77	2.35	4.12	57	0.18	0.61	0.16	0.22	0.38	58

TC, Total cholesterol as sum of free and esterified cholesterol; DTNB, disodium 5,5′-dithiobis-(2-nitrobenzoate).

plasma and of such plasma supplemented with HDL_2 or HDL_3, there was de novo synthesis of 0.4, 0.6 and 0.5 mmol/l, respectively, of cholesteryl esters after 24 hr of incubation.

Free cholesterol. In LCAT-inhibited incubation experiments there was a slight increase in the free cholesterol content of VLDL by about 0.1 mmol/l, corresponding to a decrease in free cholesterol of LDL and HDL. The most predominant decrease of free cholesterol was found in the HDL of native FED plasma, which was reduced by 60% of its total free cholesterol. The decreases in free cholesterol content of the HDL_2 and HDL_3 lipoprotein fractions in corresponding incubations were only about 20%, but the absolute decrease was similar to the decrease in free cholesterol of the HDL originating from FED plasma. Within the error of estimation, the increases in the free cholesterol content of VLDL roughly corresponded to the total decrease in this lipid of the LDL and HDL fractions.

The recovery of free cholesterol in total isolated lipoproteins after 24 hr of incubation in the presence of LCAT inhibitor ranged from 96% to 103% for all three incubation mixtures, related to the free cholesterol concentrations estimated at start of the different incubations.

In LCAT-active incubations there was a slight increase (0.08 mmol/l) in free cholesterol of the VLDL of native

FED plasma. Such plasma, however, demonstrated a large decrease (0.50 mmol/l) in free cholesterol content of its LDL fraction.

In incubation experiments with active LCAT and HDL_2 or HDL_3, no change in free cholesterol of VLDL was found. The decrease in this lipid of the corresponding LDL fractions was about 0.3 mmol/l.

In all experiments, there was a decrease in the free cholesterol concentration of the HDL fractions. The relative reductions of free cholesterol of the HDL fractions of native, HDL_2- and HDL_3-supplemented FED plasma were about 100%, 50% and 50%, respectively.

Altogether, free cholesterol decreased in native FED plasma and the plasma supplemented with HDL_2 or HDL_3 by 0.6, 0.4 and 0.4 mmol/l, respectively, after 24 hr incubation when LCAT was active. The decrease in free cholesterol of these plasmas was mainly associated with a decrease in this lipid of LDL and HDL.

Total cholesterol. In experiments with LCAT inhibitor, the total cholesterol content of VLDL of all different incubations increased by 0.4–0.5 mmol/l. There was a concomitant 0.2–0.4 mmol/l decrease in total LDL cholesterol. Also, total cholesterol decreased in HDL of the different mixtures. The total cholesterol of the FED HDL decreased by 0.06 mmol/l, corresponding to a reduction of about 50% of total cholesterol initially present. In incubations with HDL_2 this fraction lost 0.17 mmol/l of total cholesterol, whereas in experiments with HDL_3 the loss increased to 0.23 mmol/l, both values representing total HDL cholesterol reduction by 40%. In all LCAT-inhibited experiments, the increases in total cholesterol of VLDL were balanced by the sum of the decreases in this lipid of LDL and HDL.

In LCAT-active incubations there was also an increase in VLDL total cholesterol of about 0.3–0.4 mmol/l; that value is somewhat smaller than that obtained when the enzyme was inhibited. In LDL of native FED plasma there was a corresponding decrease (0.4 mmol/l) in total LDL cholesterol, but a higher reduction (0.09 mmol/l) of its total HDL cholesterol than was seen with LCAT inhibitor. This figure, however, which has a large error of estimation due to the low cholesterol concentration, corresponds to an 87% reduction of total HDL cholesterol.

In the incubations with HDL_2 and HDL_3 the decreases in total cholesterol of these fractions were 0.20 and 0.28 mmol/l, respectively, yielding almost the same values as obtained for these lipoprotein fractions in the presence of the LCAT inhibitor DTNB. The decrease in total cholesterol content of HDL_2 and HDL_3 balanced the increase in total VLDL cholesterol in LCAT-active incubations, whereas the total cholesterol contents of the corresponding LDL fractions were essentially unchanged. The recovery of total cholesterol after 24 hr of incubation, estimated in total lipoproteins, as compared to concentrations at start of incubation ranged from 95% to 106% in all experiments with and without LCAT inhibitor.

Cholesteryl ester percentage. The cholesteryl ester percentage increased from 50% to 60% in VLDL of all incubation mixtures when LCAT inhibitor was present. However, in the corresponding LDL fractions there were virtually no changes of the relative amounts of cholesteryl ester showing a value of ca. 56%.

The cholesteryl ester percentages of FED HDL, normal HDL_2 and HDL_3 are close to 20%, 70% and 80%, respectively. It was not possible to accurately estimate changes of the cholesteryl ester percentage of HDL in incubation experiments with native FED plasmas. However, there was a tendency toward increase in relative cholesteryl ester content (to about 50%) due to a decrease in free cholesterol of the FED HDL, which was not associated with an increase in cholesteryl ester content. In the HDL fractions of the plasmas supplemented with HDL_2 or HDL_3, the cholesteryl ester percentage decreased from 56% to 46% and 69% to 58%, respectively, upon incubation with LCAT inhibitor.

When LCAT was active the cholesteryl ester percentage of VLDL also increased in all experiments from about 50% to 65%, which is a little greater increase than that obtained in the corresponding incubation with LCAT inhibitor.

In incubation mixtures with active LCAT, there was also an increase in the cholesteryl ester percentage of the LDL lipoproteins from about 55% to 65%.

At the start of incubation of FED plasma supplemented with normal HDL_2, the mean cholesteryl ester percentage of the combined HDL_2 and FED HDL originally present in such plasma was 57%. After a 24-hr reaction this percentage increased to 68%, which is the normal value for native HDL_2.

In incubation experiments with active LCAT and HDL_3, there was no change of the cholesteryl ester percentage, showing a starting value of 68%, representing a mean of the added HDL_3 and of autologous FED HDL.

Phospholipids. When whole native FED plasma and such plasma supplemented with normal isolated HDL_2 or HDL_3 was incubated with the LCAT inhibitor DTNB, followed by isolation of VLDL, LDL and HDL and estimation of their phospholipid contents, these lipids were recovered in 96% to 104% yields, calculated from the phospholipid concentrations at start of the incubations.

In these three LCAT-inhibited plasma mixtures, the phospholipid contents of their VLDL fractions virtually did not change on incubation for 24 hr. In experiments with native plasma the phospholipid content of LDL increased by 0.17 mmol/l, which corresponded to a 0.12 mmol/l decrease in the plasma HDL phospholipid content, the latter decrease representing a reduction by 52% of the initial HDL phospholipid concentration.

HDL_2-supplemented FED plasma demonstrated a slight increase in phospholipid content of LDL corresponding to a possible slight decrease in this lipid of the HDL fraction, in the presence of LCAT inhibitor. In HDL_3-supplemented plasma, similar slight changes of phospholipids of LDL and HDL occurred on incubation with LCAT inhibitor.

The concentration of phospholipids decreased by approximately 0.15 mmol/l in each of the VLDL, LDL and HDL fractions of all three incubation mixtures after a 24-hr incubation under conditions where LCAT was active. The total loss of phospholipids estimated in total lipoproteins isolated from each incubation mixture after 24 hr of incubation with active LCAT was about 0.45 mmol/l.

Particle size pattern of HDL. The HDL fractions of all incubation mixtures were isolated at 0 hr and after 7.5 and 24 hr of incubation, and their particle size patterns were analyzed by polyacrylamide gradient gel electrophoresis and staining for proteins. The results are

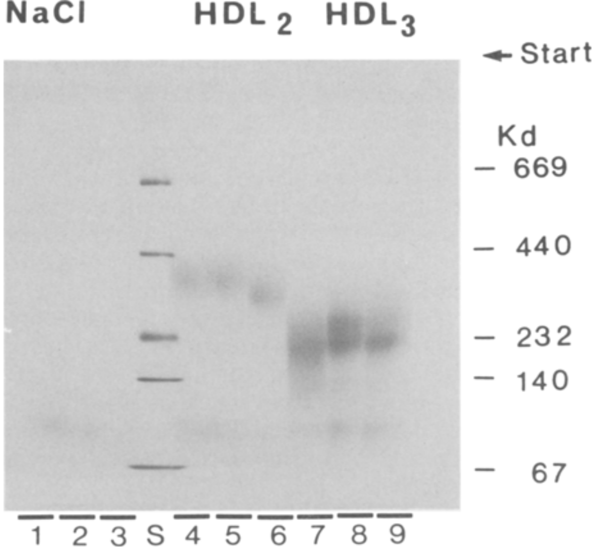

FIG. 1. Electrophoretogram of polyacrylamide gradient gel electrophoresis of the high density lipoprotein (HDL) fraction (d = 1.063–1.21 kg/l) isolated after 0, 7.5 and 24 hr of incubation at 37 C of native fish-eye disease (FED) plasma and such plasma supplemented with isolated normal HDL_2 or HDL_3. Zones are stained by Coomassie Brilliant Blue. 1–3, Native FED plasma incubated for 0, 7.5 and 24 hr, respectively; 4–6, FED plasma supplemented with HDL_2 incubated for 0, 7.5 and 24 hr, respectively; 7–9, FED plasma supplemented with HDL_3 incubated for 0, 7.5 and 24 hr, respectively; s, molecular mass markers same as in ref. 7; Kd, kilodalton.

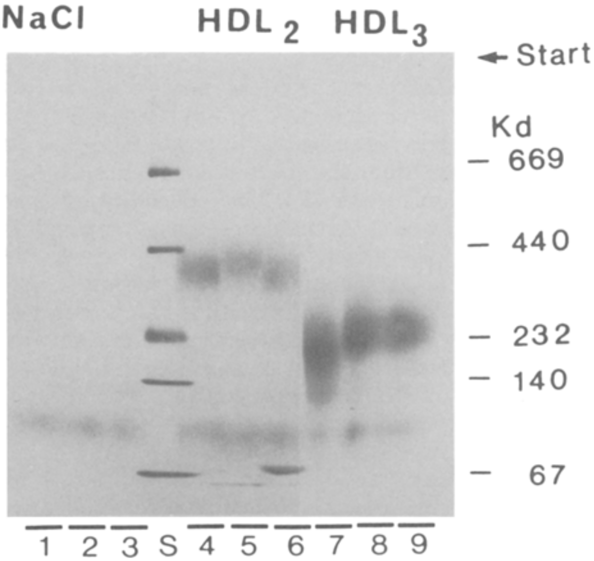

FIG. 2. This figure represents the same incubation experiments as in Fig. 1, but performed in the presence of the LCAT inhibitor disodium 5,5′-dithiobis-(2-nitrobenzoate).

shown in Figures 1 and 2. These figures demonstrate that the native FED plasma HDL and this HDL in plasma supplemented with HDL_2 almost disappeared after 24 hr of incubation with active LCAT. At supplementation of plasma with HDL_3, however, FED HDL seemed less affected by incubation or its position on the electrophoretogram might be superimposed by small HDL particles produced from the added isolated HDL_3 particles as a result of the incubation process.

In the corresponding incubations with LCAT inhibitor, FED HDL did not seem to be affected by the incubation process. The small FED HDL particles did not seem to fuse with the added normal HDL_2 or HDL_3, but remained as homogenous particle populations. In all experiments with and without LCAT inhibitor there was an incubation-induced increase of the size of the HDL_3 particles related to the added HDL_3 preparation. By contrast, after 24 hr of incubation the HDL_2 fraction was unaffected or slightly reduced in particle size.

DISCUSSION

It has been postulated that plasma cholesteryl esters are primarily formed in HDL_3 or in a subfraction of these lipoproteins by the action of LCAT on the free cholesterol at the HDL particle surface. Formed esters are subsequently distributed to acceptor lipoproteins of lower densities (13) by active lipid transfer proteins exchanging lipids between VLDL, LDL and HDL (for review see 14–16).

However, evidence has been presented that in FED plasma almost all cholesteryl esters are synthesized within the β-lipoproteins (VLDL and LDL) by the direct action of LCAT activity (β-LCAT) (3,4). This plasma virtually lacks the property to esterify the free cholesterol of isolated normal HDL_3 as well as that of the very small, but spherical, free cholesterol-rich HDL particles present in both FED and LCAT-deficient plasma (2,7,17). These latter lipoproteins have been demonstrated to be excellent substrates for normal LCAT (3,17,18). As β-LCAT also could be demonstrated in HDL-depleted normal plasma to esterify the free cholesterol of both autologous and homologous combined VLDL and LDL (4), we introduced the hypothesis that there normally exist two LCAT activities denoted α- and β-LCAT in normal plasma, specific for HDL and combined VLDL and LDL, respectively (3). According to this concept fish-eye disease is an α-LCAT deficiency syndrome. The apparent deficiency of the HDL cholesterol esterifying activity (α-LCAT) might be explained in several ways. One obvious possibility is that one of two isoenzymes of LCAT, which according to our hypothesis is present in normal plasma, is lacking in FED plasma. A second possibility would be that FED β-LCAT is a mutant of a normal LCAT, resulting in an enzyme lacking activity for HDL cholesterol but with a functioning β-lipoprotein esterifying activity. In the present study a possible role of a lacking or impaired cholesteryl ester transfer or lipid transfer activity has been considered as yet another cause for the apparent lack of α-LCAT.

It has been suggested that LCAT exists in plasma in a complex with apolipoprotein A-I, which is qualitatively normal in fish-eye disease (19), and apolipoprotein D, which has been ascribed the role of a cholesteryl ester transfer protein residing in HDL (5). The observed preference of normal LCAT for HDL_3 in incubations in vitro with isolated lipoproteins was suggested to be an artifact induced by the ultracentrifugation isolation technique (5). It was proposed that instead of acting directly on lipoprotein particles, LCAT esterifies free cholesterol present in

an LCAT-apo A-I-apo D complex and that all major lipoprotein fractions of plasma could act as recipients of the enzymatically synthesized cholesteryl esters (5).

Evidently combined VLDL and LDL functions as substrate for an active LCAT (β-LCAT) in FED plasma. It has also previously been demonstrated that cholesteryl ester transfer activity in lipoprotein-depleted FED plasma is normal (6) and that this plasma contains normal apolipoproteins A-I and D, although in strongly reduced concentrations compared to normal plasma (2,19). Thus the basic qualitative requirements for a functioning cholesterol-esterifying complex (5) as described above seem fulfilled in fish-eye disease.

However, as FED HDL total cholesterol and relative amount of cholesteryl esters are reduced by 90% and 75%, respectively, in plasma, relative to what is normal, it has not been possible to confirm in vitro with autologous lipoproteins a normal cholesteryl ester transfer from HDL to VLDL or LDL.

Thus this transfer activity together with triglyceride and phospholipid transfer activities were measured in the present study as net mass lipid transfer between VLDL, LDL and HDL in whole FED plasma supplemented with normal isolated HDL_2 or HDL_3. The addition of normal HDL fractions to such plasma would favor the formation of a postulated LCAT-cholesteryl ester transfer protein complex, normalizing the plasma concentrations of both apolipoprotein A-I and D and other unknown potentially important apolipoprotein cofactors. Also subfractions of normal HDL_3 would have a favorable structure for initiation of cholesterol esterification (14,15) by the LCAT present in FED plasma, possibly induced by isolation of HDL_3 by preparative ultracentrifugation (5,16) as discussed above. In addition the increase in the cholesteryl ester content of the plasma HDL_2 or HDL_3 fraction to a normal level made it possible to study its change with time of incubation. The results from the present experiments with FED plasma supplemented with isolated normal HDL_2 or HDL_3 and incubated in the presence of the LCAT inhibitor DTNB clearly demonstrate a net mass reduction of the cholesteryl ester contents of both HDL_2 and HDL_3 and of LDL accompanied by an increase in these lipids in VLDL. There was a concomitant decrease in the triglyceride content of VLDL associated with an increase in this lipid in HDL_2 or HDL_3 and in LDL. This indicates a functioning cholesteryl ester transfer from HDL to lipoproteins of lower densities, in agreement with what has been observed to occur in normal plasma (13–16,20). From the values for cholesteryl ester content of the HDL fraction of FED plasma supplemented with HDL_2 or HDL_3 at 0 hr and after incubation for 7.5 hr in the presence of DTNB, which inhibits LCAT (Table 3), a cholesteryl ester transfer rate of 16 μmol cholesteryl ester $\times$ hour^{-1} $\times$ liter plasma^{-1} may be calculated for the direction HDL to lipoproteins of lower densities. This figure is well within the range of the corresponding cholesteryl ester transfer rates obtained with whole plasma of normolipidemic subjects, which ranged from 12 to 40 (mean of 24) μmol cholesteryl ester $\times$ hour^{-1} $\times$ liter plasma^{-1}, and higher than those of hypertriglyceridemic subjects with documented vascular disease, whose rates varied within 0 to 15 (mean of 9) μmol $\times$ hour^{-1} $\times$ liter plasma^{-1}, as reported by Fielding et al. (21).

The concentrations of free cholesterol and phospholipids of the FED HDL decreased in incubations with LCAT inhibitor and native FED plasma. The quantitative yields of the lipids of whole plasma after incubation, however, indicate that this lipoprotein partly fuses or is taken up by the other lipoproteins in the plasma.

Also in LCAT-active incubations there was an evident cholesteryl ester transfer between added HDL_2 or HDL_3, and the other lipoproteins in the incubation mixtures balanced by triglyceride transferred in an expected counterflow direction as described above. An increase in total cholesteryl ester content was observed in such plasma mixtures that were of the same order of magnitude as previously reported for native FED plasma (2). Concomitantly the total phospholipid content of the plasma mixture decreased stoichometrically with the synthesis of new cholesteryl esters. The decrease of free cholesterol was most apparent in the LDL fraction, whereas it remained practically unchanged in VLDL. Phospholipids decreased in both VLDL and LDL and in added HDL_2 or HDL_3 by approximately the same molar amount when LCAT was active. In native FED plasma the degradation of its small HDL particles was pronounced, as estimated by a decrease in major lipid components. In addition polyacrylamide gradient gel electrophoresis showed a fading of the stain of the zone corresponding to the FED HDL particle population in LCAT-active incubations with native FED plasma and in such plasma supplemented with HDL_2. There was no degradation of FED HDL detectable by gradient gel electrophoresis in experiments with HDL_3 or when incubations were performed with LCAT inhibitor. However, both HDL_2 and HDL_3 have been demonstrated to have an inhibitory effect on β-LCAT, with the latter HDL subfraction being the strongest inhibitor of the fish-eye disease enzyme (Holmquist, L., and Carlson, L.A., submitted for publication).

The added HDL_3 particles were enlarged on incubation when LCAT was either active or inhibited, whereas the HDL_2 fraction was less affected; this is in agreement with previous findings from the effect of incubation on normal plasma HDL particle size (22). There also seemed to be an increase in the amount of small particles accompanying the enlargement of the added HDL_3 particles on incubation (Fig. 1), which is consonant with the results reported by Nichols et al. (23) for incubation experiments with HDL studied by polyacrylamide gradient gel electrophoresis.

De novo synthesis of cholesteryl esters in the HDL fractions could not be detected in any incubation experiment, although both β-LCAT and cholesteryl ester transfer activities of FED plasma were active, resulting in an increase in the plasma cholesteryl ester content of about 0.5 mmol/l. On incubation, the cholesteryl ester content of added normal HDL_2 or HDL_3 decreased with the same value whether LCAT was active or inhibited. The polyacrylamide gradient gel electrophoresis experiments indicate that the small-sized FED HDL particles in the incubation mixture did not increase in particle size, in contrast to what they do when acted upon by normal LCAT, which concomitantly increased their relative cholesteryl ester content from about 20% to 100% (3).

The zone on the polyacrylamide gradient gel electrophoretogram corresponding to FED HDL almost disappeared after 24 hr of incubation in experiments with native FED plasma and such plasma supplemented with

normal HDL_2. This phenomenon was observed earlier but was less pronounced in undiluted FED plasma (3), which had a somewhat higher amount of HDL than plasma in the present experiments. The fading of HDL-stainable material thus does not seem to be an artifact of the electrophoresis, but might be a result of reduction of HDL particle size or increase in density following particle lipid degradation (Table 2). This should give rise to loss of HDL in the 1.063-1.21 kg/l fraction on ultracentrifugation.

All the results of this study indicate that esterification of cholesterol in FED plasma occurs in combined VLDL and LDL, as previously suggested by an active β-LCAT (3), even during conditions that would favor primary esterification of the free cholesterol in normal HDL subfractions.

These results also favor the hypothesis that there might exist α- and β-LCAT activities with different substrate specificities, the α-LCAT being deficient in fish-eye disease.

Recent experiments have further demonstrated that FED HDL particles are normalized in respect to both particle size and cholesteryl ester content by total lipoprotein-depleted pig plasma (24) which is deficient of cholesteryl ester transfer activity (16). This further supports the view that the abnormally small-sized and cholesteryl ester-poor FED HDL are the result of a specific lack of an LCAT enzyme activity rather than impaired lipid transfer activities or lipid acceptor properties of the plasma lipoproteins.

ACKNOWLEDGMENTS

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Periodate-Induced Lipid Oxidation of Erythrocyte Membranes

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Exposure of human erythrocyte ghosts to 0.2-5 mM periodate at 0 C for 15 min resulted in an increase of thiobarbituric acid-reactive substances and fluorescent materials in the membrane. This increase was suppressed by radical scavengers, butylated hydroxytoluene and thiourea, indicating that periodate caused lipid oxidation of ghosts. A role of hemoglobin in the periodate-induced lipid oxidation of ghosts was suggested by the fact that the oxidation was augmented by hemoglobin and inhibited by an iron chelator, desferrioxamine. Treatment of ghosts with periodate caused membrane protein cross-linking with and without disulfide bridge. Erythrocytes were also susceptible to lipid oxidation by periodate, but only disulfide-mediated protein cross-linking was observed. Erythrocytes treated with neuraminidase or trypsin were less susceptible, but those treated with neuraminidase along with galactose oxidase were nearly as susceptible as untreated cells. The effect of galactose oxidase was diminished by reduction of the enzyme-treated cells with borohydride. These results indicate that the aldehyde moieties generated by periodate at the sialyl residues or those generated by galactose oxidase at the terminal galactosyl or *N*-acetyl galactosaminyl residues of the membrane glycoconjugates play a stimulating role in the periodate-induced membrane lipid oxidation.
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It has been demonstrated that periodate treatment of lymphocytes (1-4), macrophages (2) and erythrocytes (4-6) induces a variety of functional changes in these cells. For example, periodate treatment of erythrocytes renders them susceptible to macrophage recognition (5) and cytolysis by lymphocytes (4). It has been known that the sialyl residues of glycoproteins of the cell membranes are readily oxidized to the aldehyde-bearing derivatives by periodate (7,8), and most of the functional alterations of the cell membranes have been ascribed to the oxidation of the sialyl residues. Protein is also susceptible to periodate and affords cross-linked proteins with disulfide bridge (6,9). Reversible formation of aqueous channels or pores in the erythrocyte membrane by periodate is regarded as a consequence of the disulfide bridge formation (6).

In view of a possible involvement of lipid oxidation in the periodate-induced damage and functional changes of the cell membranes, it is important to clarify the characteristic nature of lipid oxidation of the cell membranes by periodate. We have investigated the reaction of human erythrocytes and ghosts with periodate. We will demonstrate here that extensive lipid oxidation takes place both in erythrocytes and in ghosts and will show that sialyl residues of the membrane glycoconjugates affect the periodate-induced lipid oxidation of the membrane.

MATERIALS AND METHODS

Materials. Sodium metaperiodate, thiobarbituric acid (TBA) and thiourea were obtained from Wako Pure Chemical Industries. Butylated hydroxytoluene (BHT) and desferrioxamine (Desferal) were from Nikki Universal Company and Ciba-Geigy, respectively. Linoleic acid and methyl linoleate were from Nippon Oil and Fats Company. Oleic acid and methyl linolenate were from Nakarai Chemicals. γ-Linolenic acid was from Sigma Chemical Co. Neurminidase (E.C.3.2.1.18, *Vibrio cholerae*) was from Behringerke AG. *N*-Acetylneuraminic acid (NANA, type VI) and soybean trypsin inhibitor were from Sigma. Trypsin (bovine pancreas) and galactose oxidase (E.C.1.1.3.9, *Dactylium dendroides*) were from Worthington Diagnostic Systems. Hemoglobin was purified from the human erythrocyte lysate by CM Sephadex C-50 ion exchange column chromatography as described elsewhere (10).

Erythrocytes and ghosts. Human venous blood was collected from a healthy donor using citrate-phosphate-dextrose as an anticoagulant and was stored at 4 C for use within a few days. The blood was centrifuged to remove plasma and buffy coats. Erythrocytes were washed 4 times with isotonic saline and resuspended in Dulbecco's phosphate-buffered saline (DPBS, pH 7.2). Erythrocyte ghosts were isolated according to the method of Dodge et al. (11) and were stored at −20 C in aliquots for use. Hemoglobin content in the ghosts was usually 1-3% of the total proteins as determined by the method reported (11). Protein concentrations were determined by the method of Lowry et al. (12).

Measurement of sialic acid. Free sialic acids were determined by the method of Warren (13). To a sample solution (0.2 ml) was added 0.1 ml of 0.2 M sodium metaperiodate in 9 M phosphoric acid, and the solution was kept at 22 C for 20 min. After destroying an excess amount of periodate by addition of 1.0 ml of 10% sodium arsenite solution in 0.05 M sulfuric acid-0.5 M sodium sulfate, the mixture was heated with 3.0 ml of 0.6% TBA in 0.5 M sodium sulfate at 100 C for 15 min. After cooling, the mixture was extracted with 4.3 ml of cyclohexanone, and the absorbance at 549 nm of the extract was recorded. The standard NANA solution was similarly treated as a control. For the determination of the total sialic acid contents of intact erythrocytes and ghosts, the cells were heated in 0.05 M sulfuric acid at 80 C for 1 hr, and the released sialic acid was purified by successive passing through Dowex 50 (H^+) and Dowex 1 (formate) columns (13).

To estimate the amounts of the unoxidized sialic acid residues in the periodate-treated ghosts, the control and the oxidized ghosts (1-3 mg protein/ml) were treated with neuraminidase (25 mU/ml) at 37 C for 30 min, and the released sialic acid in the supernatant was determined.

For measurement of the amounts of sialic acid residues released from the neuraminidase-treated erythrocytes, the supernatant was directly subjected to sialic acid determination. The supernatant of the trypsin-treated erythrocytes was successively incubated with neuraminidase (25 mU/ml) at 37 C for 3 hr to release free sialic acid for assay.

Measurement of lipid oxidation. (a) Formation of TBA-reactive substances. Lipid oxidation of the membranes was assessed by measuring TBA-reactive substances (TBA-RS) according to the method of Buege and Aust

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(14). Briefly, 1.0 ml of a membrane suspension (2–3 mg protein/ml) was mixed with 2.0 ml of a solution of 0.375% TBA-trichloroacetic acid (TCA)-0.25 N HCl containing 0.01% BHT, and the mixture was heated at 100 C for 15 min. After cooling, the mixture was centrifuged, and the absorbance at 532 nm of the supernatant was measured. The amount of TBA-RS was expressed as the absorbance at 532 nm for the membrane specimen containing 2 mg protein.

Measurement of TBA-RS of fatty acids and their methyl esters was carried out as follows. The sample was heated in a mixture of 1.0 ml of 0.5% TBA and 2.9 ml of 0.5% TCA at 60 C for 90 min under anaerobic conditions. After cooling, the mixture was extracted with 3.0 ml of chloroform to give a clear aqueous phase. The absorbance at 532 nm of the aqueous phase was recorded.

(b) Formation of fluorescence. Formation of fluorescent substances in the membranes was assessed as an index of lipid oxidation (15). For this purpose, the membranes were solubilized in 5% sodium dodecyl sulfate (SDS)–10 mM sodium phosphate buffer (pH 7.0). Fluorescence intensities were measured at an excitation maximum of 354 nm and an emission maximum of 437 nm. Relative fluorescence intensity of the solubilized membrane against 0.1 μM quinine sulfate was expressed for the protein concentration of 10 mg/ml.

SDS polyacrylamide gel electrophoresis. SDS gel electrophoresis was performed by the method of Laemmli (16) in a 7.5% gel. Ghosts were solubilized in the Laemmli sample buffer. For preparation of nonreduced samples, 2-mercaptoethanol was omitted from the sample buffer. Sample containing 40 μg protein was loaded for each lane. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. Major protein bands of erythrocyte membrane are numbered according to Steck (17).

Treatment of erythrocytes and ghosts with periodate. A suspension of intact or enzyme-treated erythrocytes (hematocrit value 20%) was mixed with an equal volume of freshly prepared 0.4–10 mM sodium metaperiodate solution in DPBS. The reaction was performed at 0 C for 15 min in the dark with occasional mixing. The cells were recovered by washing four times with DPBS at 0–4 C. The ghosts were prepared by hypotonic hemolysis using standard procedures (11) and were resuspended in 10 mM phosphate buffer (pH 7.0) for subsequent analysis.

Reaction of ghosts, intact or from the enzyme-treated erythrocytes, with periodate was carried out by mixing the ghost suspension (5 mg protein/ml) in 10 mM phosphate buffer (pH 7.0) with an equal volume of the periodate solution. After incubation at 0 C for 15 min in the dark, the ghosts were recovered by centrifugation (15,000 × g, 20 min) and washed four times with 10 mM phosphate buffer (pH 7.0) at 0–4 C. The recovered ghosts were resuspended in the same buffer for subsequent analysis.

Treatment of erythrocytes with enzymes. An erythrocyte suspension (hematocrit value 50%) in DPBS was incubated with neuraminidase (25 mU/ml), trypsin (0.1 mg/ml) or neuraminidase (25 mU/ml) plus galactose oxidase (10 U/ml) at 37 C for 30 min. The trypsinized cell suspension was incubated for a further 3 min with trypsin inhibitor (0.2 mg/ml). The cells and the supernatant were separated by centrifugation. The supernatant was subjected to sialic acid determination. The cell pellet was washed three times with DPBS to give the enzyme-treated erythrocytes. The ghosts were prepared by using standard procedures (11).

RESULTS

Human erythrocyte ghosts were treated with 5 mM periodate at 0 C for 15 min. Loss of the sialic acid residues was assessed by TBA reagent according to the method of Warren (13) (Fig. 1A). Sialic acid that can be released from untreated ghosts by neuraminidase (90% of the total sialic acid) formed the color with a maximum absorption at 549 nm, which was identical with that of the standard NANA. The supernatant of the enzyme digest of the periodate-treated ghosts exhibited an absorbance at 549 nm, as low as 5% of that of the untreated ghosts. Thus, most of the sialic acid residues on the ghosts were lost by the periodate treatment. They were oxidized probably to 8- or 7-carbon analogues bearing aldehyde moieties (7,8).

TBA-RS of the ghosts were measured as an index of lipid oxidation (14) (Fig. 1B). Untreated ghosts produced a color with a maximum absorption at 532 nm, which was identical with that of the standard malonaldehyde. The periodate-treated ghosts revealed much higher absorbance at 532 nm, indicating that formation of TBA-RS was accelerated by the treatment. This substantial increase in TBA-RS may be due to lipid oxidation of the ghosts.

To examine the ability of periodate to oxidize unsaturated fatty acids and their methyl esters, emulsified fatty acids were incubated with periodate. While incubation of methyl linoleate with 5 mM periodate at 0 C for 15 min under aerobic conditions showed no significant increase in TBA-RS, incubation of linoleic acid, methyl linoleate, γ-linolenic acid and methyl linolenate with 50 mM periodate at 37 C for 48 hr resulted in a substantial increase in TBA-RS (Table 1). Periodate at the relatively high concentration can accelerate oxidation of the unsaturated fatty acids.

Ghosts were treated with various concentrations of periodate at 0 C for 15 min. TBA-RS accumulated in the

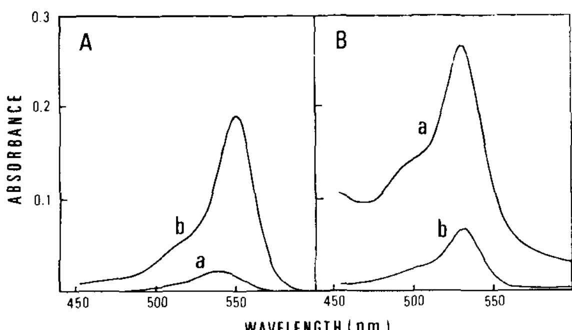

FIG. 1. Absorption spectra of thiobarbituric acid (TBA) color in sialic acid measurement (A) and TBA-reactive substances (RS) (B) of the periodate-treated ghosts. A: Spectra of TBA color from the sialic acid residues released from the ghosts by neuraminidase. B: Spectra of TBA-RS due to lipid oxidation in the ghosts. Absorbance obtained for the ghosts containing 2 mg protein was presented. a) Ghosts (2.5 mg protein/ml) treated with 5 mM periodate at 0 C for 15 min. b) Untreated ghosts, which contained 30 μg total sialic acid/mg protein and released 27 μg sialic acid by neuraminidase digestion.

TABLE 1

Oxidation of Unsaturated Fatty Acids by Periodate[a]

Unsaturated fatty acid	Thiobarbituric acid–reactive substances ($A_{532\ nm}$)[b]		
	No incubation	Incubated without periodate	Incubated with periodate
Oleic acid	0.000	0.000	0.000
Linoleic acid	0.009	0.053	0.088
Methyl linoleate	0.019	0.044	0.279
γ-Linolenic acid	0.491	0.579	0.665
Methyl linolenate	1.93	14.0	33.3

[a]An emulsified mixture (10 ml) of an unsaturated fatty acid or its methyl ester (20 mM) and periodate (50 mM) was incubated at 37 C for 48 hr in the dark in a loosely capped tube. At the end of the incubation, 1.7 ml of a solution of 10% sodium arsenite–0.05 M sulfuric acid–0.5 M sodium sulfate was added to destroy the residual periodate, and the mixture was extracted with 1.5 vol chloroform. Each 2 ml of the chloroform layer was evaporated to dryness to determine thiobarbituric acid–reactive substances.

[b]Values derived from 20 μmol of each unsaturated fatty acid are shown.

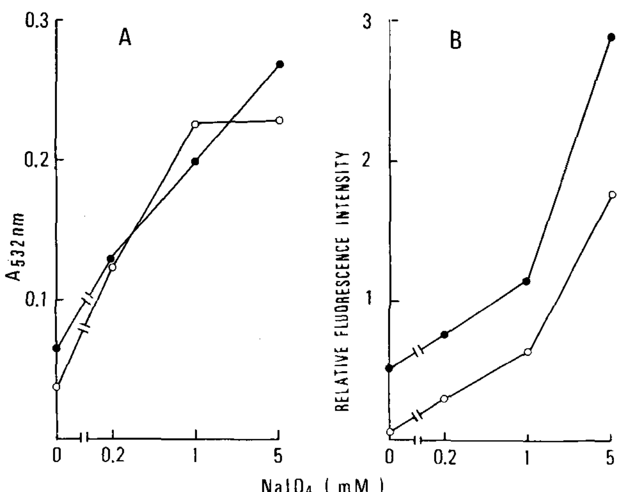

FIG. 2. Formation of thiobarbituric acid–reactive substances (A) and fluorescence (B) in the periodate-treated ghosts and erythrocytes. ●, Suspension of ghosts (2.5 mg protein/ml) in periodate solution at the indicated concentration was incubated at 0 C for 15 min. ○, Erythrocyte suspension (10%) in periodate solution at the indicated concentration was incubated at 0 C for 15 min. The ghosts were prepared by hypotonic hemolysis for the assay. $A_{532\ nm}$ is expressed for the amount of ghosts containing 2 mg protein.

membranes at the periodate concentrations above 0.2 mM (Fig. 2A). Formation of fluorescence was examined as another index of lipid oxidation (15). The maximum wavelengths of the fluorescence spectra of the membranes were 354 (excitation) and 437 nm (emission), similar to those of the erythrocyte membranes oxidized by organic hydroperoxides (18) and 13-linoleic acid hydroperoxide (19). The fluorescent substances accumulated in the membranes at the periodate concentrations above 0.2 mM (Fig. 2B). The fluorescent substances distributed in both

TABLE 2

Inhibition of Periodate-Induced Thiobarbituric Acid-Reactive Substances (TBA-RS) and Fluorescence Formation in Ghosts[a]

Treatment of ghosts	TBA-RS[b] ($A_{532\ nm}$/ 2 mg protein)	Relative fluorescence intensity[b]
Control (no treatment)	0.029 (10)	0.88 (23)
Periodate	0.286 (100)	3.83 (100)
Periodate in the presence of		
Butylated hydroxytoluene (0.1 mM)	0.142 (50)	1.11 (29)
Thiourea (30 mM)	0.032 (11)	0.90 (23)
Desferrioxamine (0.5 mM)	0.042 (15)	0.92 (24)

[a]Ghosts (2.5 mg protein/ml) were treated with 1 mM periodate at 0 C for 15 min in the presence or absence of the indicated compounds.

[b]Numbers in parentheses are TBA values or fluorescence intensities expressed as percentage of the values of the ghosts treated with periodate alone.

lipid and protein fractions (data not shown). The periodate-induced TBA-RS formation in ghosts was inhibited partially by BHT and completely by thiourea and desferrioxamine (Table 2). Formation of fluorescence was inhibited completely by these reagents (Table 2). These inhibitory effects are probably due to specific properties of the compounds—i.e., BHT is an antioxidant of lipid oxidation, thiourea a hydroxyl radical scavenger and desferrioxamine a potent iron chelator (18,20)—and not to a nonspecific reaction between these compounds with periodate. This is supported by the fact that the periodate concentration is 10-fold that of BHT (Table 2), whereas in the case of thiourea and desferrioxamine, it was observed that urea, an analogous compound of thiourea, and desferrioxamine saturated with Fe^{3+} were ineffective under the same conditions. These results indicate that the radical reactions are involved in the periodate-induced TBA-RS and fluorescence formation and substantiate that lipid oxidation is induced in ghosts by periodate. Inhibition of the periodate-induced lipid oxidation of ghosts by desferrioxamine suggests an essential role for hemoglobin in the reactions (21,22). The periodate-induced TBA-RS and fluorescence formation in ghosts increased with the concentrations of exogenously added hemoglobin (Fig. 3). In this case, hemoglobin alone did not induce lipid oxidation. It is evident that the periodate-induced lipid oxidation of ghosts was catalytically accelerated by hemoglobin.

Cross-linking of the membrane proteins was analyzed by SDS gel electrophoresis in the presence or absence of 2-mercaptoethanol (Fig. 4). Under nonreducing conditions (Fig. 4A), bands 1, 2, 3, 4.1, 4.2 and 5 diminished as the periodate concentration increased (lanes 2–4). At 5 mM periodate, these bands totally disappeared (lane 4). As these bands diminished, higher molecular weight proteins appeared near the top of the gel (lanes 2–4). There were no remarkable differences in the protein band patterns before (Fig. 4A) and after (Fig. 4B) reduction with 2-mercaptoethanol. Hence, extensive protein cross-links other than disulfide bridges were formed by the periodate treatment. Effects of BHT, thiourea and desferrioxamine on the protein cross-linking were analyzed under reducing

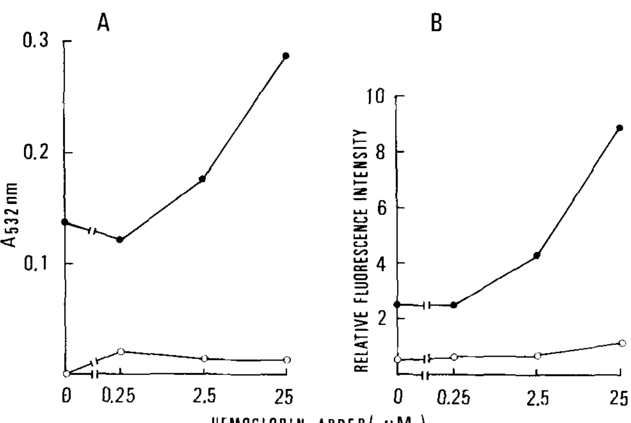

FIG. 3. Effect of hemoglobin on periodate-induced thiobarbituric acid-reactive substances (A) and fluorescence (B) formation in the ghosts. Ghosts (2.5 mg protein/ml) were treated with (●) or without (○) 1 mM periodate in the presence of increasing amounts of hemoglobin at 0 C for 15 min. The ghost preparation originally contained hemoglobin (2.7% of the total protein), which corresponded to 1 μM under the reaction condition. $A_{532\ nm}$ is expressed for the amount of ghosts containing 2 mg protein.

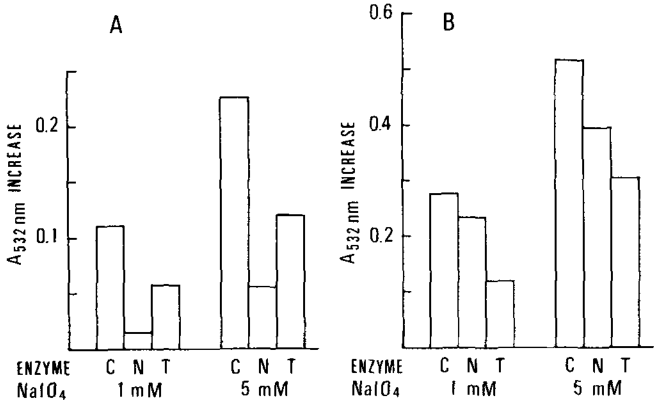

FIG. 5. Effects of neuraminidase and trypsin treatment of erythrocytes on the periodate-induced thiobarbituric acid-reactive substances formation in erythrocytes (A) and ghosts (B). An erythrocyte suspension (50%) was preincubated with neuraminidase (N), trypsin (T) or without the enzymes (C), as described in Materials and Methods. The neuraminidase (N)- and trypsin (T)-treated cells contained 40% and 64% of the original sialic acid contents, respectively. The enzyme-treated cell suspensions (10%) in periodate solution at the indicated concentrations were incubated at 0 C for 15 min. The ghosts (2.5 mg protein/ml) prepared from the preincubated erythrocytes were similarly treated with periodate. $A_{532\ nm}$ is expressed for the amount of ghosts containing 2 mg protein.

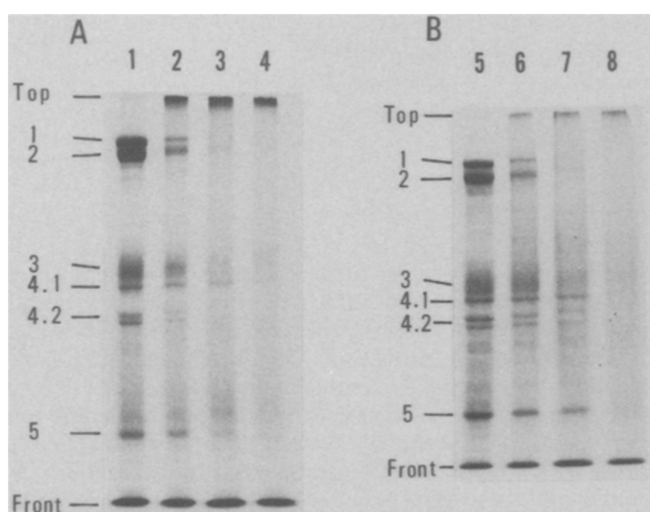

FIG. 4. SDS polyacrylamide gel electrophoresis of the periodate-treated ghosts. Electrophoresis was performed under nonreducing (A) and reducing (B) conditions. Control ghosts, lanes 1 and 5; ghosts treated with 0.2 mM periodate, lanes 2 and 6; 1 mM periodate, lanes 3 and 7; and 5 mM periodate, lanes 4 and 8. Treatment of ghosts with periodate was performed as described in the legend to Fig. 2.

conditions. All of these agents inhibited the periodate-induced loss of the protein bands and the appearance of the high molecular weight proteins, suggesting that the nonreducible cross-linking of the ghost proteins was due to lipid oxidation.

Erythrocytes were treated with periodate at 0 C for 15 min, and the modified ghosts isolated were analyzed for TBA-RS and fluorescence formation. Accumulation of TBA-RS and fluorescence increased at the periodate concentrations above 0.2 mM (Fig. 2). On an SDS electrophoresis gel under nonreducing conditions, the membrane specimen from the cells treated with 5 mM periodate indicated partial disappearance of bands 1 and 2 and concomitant appearance of the higher molecular weight proteins. Under reducing conditions, however, all the bands remained intact. The results indicate that the periodate-induced membrane protein cross-linking in erythrocytes was due to only disulfide bridge formation.

To assess the effect of oxidation of the cell surface sialyl residues, the sialyl residues were removed by neuraminidase and trypsin before exposure to periodate. Treatment with neuraminidase and trypsin removed 60% and 36% of the total sialic acid of the erythrocyte surface, respectively. The enzyme-treated erythrocytes were exposed to periodate. As shown in Figure 5A, the periodate (1 mM)-induced increase of TBA-RS of the neuraminidase-treated cells was much less than that of the untreated control cells. The periodate-induced increase of TBA-RS of the trypsin-treated cells was also lower than that of the control cells. Similar effect of the enzyme treatment was observed when these cells were exposed to 5 mM periodate. Suppression of TBA-RS formation by the enzyme treatment was also observed when the ghosts isolated from the enzyme-treated erythrocytes were exposed to periodate (Fig. 5B). These results indicate that the removal of the sialyl residues from the membranes reduced the susceptibility of erythrocytes to the periodate-induced lipid oxidation.

The aldehyde moieties produced by periodate in the sialyl residues may be responsible for the stimulatory effect in the periodate-induced lipid oxidation. The effect of aldehyde moieties produced enzymatically at the galactosyl and *N*-acetyl galactosaminyl residues of the desialylated cell surface was examined. Erythrocytes were treated with neuraminidase along with galactose oxidase, an enzyme that generates aldehyde moieties at the terminal galactosyl and *N*-acetyl galactosaminyl residues of the carbohydrate chains (23,24). The enzyme-treated cells were then exposed to periodate. As shown in Table 3, the periodate-induced TBA-RS formation of the neuraminidase-treated

TABLE 3

Effect of Galactose Oxidase Treatment of Desialylated Erythrocytes on the Periodate-Induced Thiobarbituric Acid–Reactive Substances (TBA-RS) Formation

Pretreatment[a]	TBA-RS ($A_{532\ nm}$/2 mg protein) Without periodate[b]	TBA-RS ($A_{532\ nm}$/2 mg protein) With 5 mM periodate[b]	Increase in TBA-RS (% of control)
Control	0.039 ± 0.001[c] 0.058 ± 0.002[d]	0.184 ± 0.023[c] 0.177 ± 0.010[d]	100[c] 100[d]
Neuraminidase	0.035 ± 0.001[c]	0.086 ± 0.013[c]	35[c]
Neuraminidase + galactose oxidase	0.042 ± 0.002[c] 0.047 ± 0.010[d]	0.141 ± 0.004[c] 0.142 ± 0.018[d]	68[c] 80[d]
Neuraminidase + galactose oxidase, then $NaBH_4$	0.044 ± 0.010[d]	0.089 ± 0.013[d]	38[d]
$NaBH_4$	0.046 ± 0.002[d]	0.156 ± 0.007[d]	92[d]

[a]An erythrocyte suspension (50%) was preincubated with neuraminidase or neuraminidase plus galactose oxidase or without the enzymes, as described in Materials and Methods. The residual sialic acid on the cells treated with neuraminidase and neuraminidase plus galactose oxidase were 28% and 34% of the original sialic acid contents, respectively. In experiment 2, the enzyme-treated or untreated erythrocyte suspensions (20%) were incubated with or without 5 mM $NaBH_4$ in 50 mM phosphate buffer containing 0.11 M NaCl (pH 7.5) followed by washing three times with Dulbecco's phosphate-buffered saline.

[b]The pretreated cell suspensions (10%) were incubated at 0 C for 15 min with or without 5 mM periodate. Values shown are mean ± SD of triplicate incubations.

[c]Experiment 1.

[d]Experiment 2.

cells was as low as 35% of that of untreated cells, whereas that of the cells treated with neuraminidase along with galactose oxidase was 68% and 80%. Although galactose oxidase generates hydrogen peroxide during the oxidation of the substrates (23), the peroxide generated under these conditions did not cause lipid oxidation because the enzyme treatment alone did not result in the increase in TBA-RS formation. Thus, galactose oxidase substantially compensated the loss of susceptibility of the desialylated cells to periodate. Reduction of the neuraminidase and galactose oxidase–treated cells with borohydride substantially suppressed the recovered susceptibility of the cells to periodate, while reduction of the untreated cells affected their susceptibility only slightly, indicating involvement of the aldehyde moieties generated by galactose oxidase in its effect. These results suggest that generation of aldehyde moieties at the terminal carbohydrate residues of the erythrocyte surface glycoconjugates renders the cells susceptible to periodate-induced lipid oxidation.

DISCUSSION

Periodate-treated erythrocytes are susceptible to macrophage recognition (5) and cytolysis by lymphocytes (4). To elucidate the mechanism of periodate-induced changes in biological responses, it is important to clarify the chemical changes of the membrane constituents. Periodate cleaves vicinal α-glycols (25), and sialic acid residues of the cell surface carbohydrate chains are preferentially oxidized at the low concentrations (8). The oxidized form of the sialyl residues, 8- and 7-carbon analogues, contains an aldehyde moiety at the 8 and 7 positions of the residues, respectively (7,8). Periodate also oxidizes proteins. Oxidation of tyrosine and cysteine residues of isolated spectrin by periodate at 22 C was reported (9). Heller et al. (6) reported that methionine was oxidized by the periodate treatment of erythrocytes at 0 C and that thiol groups of the membrane proteins were oxidized to disulfides, which caused an increase in membrane permeability.

In this report, we have verified the lipid oxidation of erythrocyte membranes by periodate and showed the characteristic nature of the reaction. The conversion of sialyl residues to the oxidized forms was confirmed by measurement of intact sialyl residues on the periodate-treated ghosts. Involvement of radical reactions was shown by the inhibition of TBA-RS and fluorescence formation by BHT and thiourea. Inhibition by desferrioxamine suggested that a certain iron species was essential for the periodate-induced lipid oxidation of ghosts. The iron species in ghosts is likely to be originated from hemoglobin. We have previously demonstrated that hemoglobin stimulated the 13-linoleic acid hydroperoxide-induced lipid oxidation of ghosts and that this was inhibited by desferrioxamine (21). Gutteridge (22) also has reported that desferrioxamine inhibited the methemoglobin-stimulated lipid oxidation of liposomes. The catalytic action of hemoglobin appeared to be mediated by some iron complexes released from the protein upon interaction with lipid hydroperoxide. Oxidation of unsaturated fatty acids by periodate required higher concentrations

of periodate, higher temperature and longer exposure than that of the erythrocyte membranes. The presence of hemoglobin in ghosts may be one of the factors for the higher susceptibility of ghosts to periodate-induced lipid oxidation.

SDS gel electrophoresis analysis of the periodate-treated ghosts suggested that there were two types of membrane protein cross-linking, with and without disulfide bridge. The protein cross-linking without disulfide bridge may be formed as a consequence of lipid oxidation. The protein cross-linking with disulfide bridge may be formed by a direct action of periodate to the proteins or by an indirect action via lipid oxidation. SDS gel electrophoresis of the ghosts from the periodate-treated erythrocytes suggested that most membrane protein cross-links were due to disulfide bridges, which was consistent with the observation of Heller et al. (6). This may indicate that erythrocytes are less susceptible to lipid oxidation than ghosts. Cytoplasmic enzymes protective against oxidation may be involved (26). A high content of cytoplasmic hemoglobin may also protect the membrane since the high concentration of hemoglobin appears to behave as a lipid radical scavenger (21,27).

It is interesting to note that the aldehyde groups formed in the sialyl residues by periodate oxidation seem to play a stimulating role in the periodate-induced lipid oxidation. Susceptibility of erythrocytes to the periodate-induced lipid oxidation decreased when cell surface sialyl residues were removed. The removed sialyl residues may be mostly those of glycophorin, considering its high sialic acid content (28) and susceptibility to trypsin (29). The aldehyde moieties generated by galactose oxidase at the terminal galactose or *N*-acetyl galactosamine of the desialylated cell surface carbohydrate chains were also effective in stimulating the lipid oxidation. It is not known at present how the aldehyde moieties of the cell surface carbohydrate chains render the membrane susceptible to lipod oxidation by periodate. Conversion of the hydroxyl groups at the termini of the carbohydrate chains of membrane glycoproteins to aldehydes might cause conformational changes of the glycoproteins or membrane perturbations, resulting in an increased accessibility of or a decreased cellular protective function against periodate.

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Comparison of the Clearances of Serum Chylomicron Triglycerides Enriched with Eicosapentaenoic Acid or Oleic Acid

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Rat mesenteric lymph chylomicrons containing triglycerides enriched with either [^{14}C]oleic acid (OA) or [^{14}C]-eicosapentaenoic acid (EPA) were prepared by ultracentrifugation of lymph samples collected for 6 hr after a single duodenal infusion of an emulsion containing either fatty acid. These chylomicrons were injected into the jugular vein of recipient rats and, at various time intervals, blood was drawn and serum was assayed for radioactivity. In separate animals, serum lipoprotein fractions were separated by ultracentrifugation, and the redistribution of labeled fatty acid among circulating lipoproteins was determined by liquid scintillation spectrometry. When the early disappearance rates (10 min) of either total serum radioactivity or specifically the chylomicron fraction were compared, there were no differences between the groups receiving OA- or EPA-enriched chylomicrons. However, disappearance rates of EPA-enriched chylomicrons were slower than those of OA-enriched chylomicrons from 25 to 90 min. The small but significant differences in the disappearance rates for the longer time periods cannot be ascertained without further studies. At 5 min after injection of either type of chylomicron, the d < 1.006 g/ml lipoprotein fraction of serum chylomicrons and very low density lipoproteins contained almost 90% of the original radioactivity. By 240 min, when less than 2% of the radioactivity remained, this radioactivity in the d < 1.006 g/ml fraction was 43-46%, with concomitant increases in the low and high density lipoprotein fractions and in the lipoprotein-free serum. ***Lipids 22,*** **318-321 (1987).**

Relatively large amounts of dietary fish oil containing the ω-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid may reduce plasma triglyceride and cholesterol levels in humans and experimental animals (1-7), may alter hepatic lipoprotein production (8-10) and may modify prostaglandin metabolism (11-13). These responses may explain, at least in part, the lower incidence of ischemic heart disease and thrombosis in Greenland Eskimos (14), Japanese who live in fishing villages (15) and coastal-dwelling Turks (16).

Harris and Connor (17) observed that the plasma triglyceride elevation was smaller after a single dose of salmon oil than after a control (corn oil) fat dose; they suggested that this was due to a more rapid serum clearance of the ω-3 fatty acid-containing chylomicrons. However, Brockerhoff et al. (18) and Bottino et al. (19) suggested that fish oils may not be digested as efficiently as triglycerides containing more conventional fatty acids (e.g., ω-6 and ω-9 types), which could also explain the lower plasma triglyceride responses to dietary fish oils. We have found that cardiac lipoprotein lipase activity on chylomicron triglycerides enriched with EPA is comparable to that on chylomicron triglycerides enriched with oleate during recirculating rat heart perfusion (20).

The purposes of the present study were a) to compare the serum clearance of chylomicron triglycerides enriched with either [^{14}C]EPA or [^{14}C]oleic acid (OA) and b) to compare the rates and degrees of redistribution of [^{14}C]EPA and [^{14}C]OA from chylomicrons among serum lipoproteins.

MATERIALS AND METHODS

Bovine serum albumin fraction V (fatty acid-poor), sodium taurocholate and nonradioactive OA and EPA were obtained from Sigma Chemical Co. (St. Louis, Missouri). Isotopic fatty acids, labeled in the carboxyl position, were obtained from New England Nuclear (Boston, Massachusetts). Other chemicals and solvents were of the highest purity.

The test emulsions used for intraduodenal administration were prepared as described earlier (21) and included (per 1.5 ml physiological saline) 25 mg bovine serum albumin, 86 mg sodium taurocholate and 0.3 mmol OA or EPA. In addition, each emulsion contained 10 μCi of the respective [1-^{14}C]labeled fatty acid. The emulsions were prepared immediately before use and rehomogenized before each administration.

Adult male albino rats of the Wistar strain (Charles River Laboratories, Wilmington, Massachusetts), ca. 250 g, were allowed food (Purina Rat Chow, Ralston Purina Co., St. Louis, Missouri) and water ad libitum before use. Each group had four rats. The animals, under sodium pentobarbital anesthesia, were subjected to cannulation of the mesenteric lymphatic duct, and an indwelling catheter was placed in the duodenum for continuous administration of physiological saline-5% glucose (3 ml/hr). After the rats were fasted overnight, the saline-glucose infusion was interrupted, 1.5 ml of the appropriate test emulsion was infused via the duodenal catheter, and the saline-glucose infusion was reestablished. Lymph was collected on ice for 6 hr and was subjected to ultracentrifugation at d < 1.006 g/ml (3×10^6 g min) to obtain chylomicrons (21), which were purified by flotation under the same conditions. Then the distribution of chylomicron lipid fractions was determined as described previously (22).

After recipient rats were anesthetized by intraperitoneal injection with sodium pentobarbital, an indwelling catheter was inserted in the jugular vein. The chylomicron suspension (1.0-1.2 ml containing 0.012 mmol fatty acid equivalents; ca. 1×10^6 dpm) was injected and the catheter was rinsed with 1 ml saline. At 0, 2, 5, 10, 25, 60 and 90 min, 0.3 ml of blood was drawn and allowed to clot. Serum was separated by centrifugation ($100 \times$ g) and counted by liquid scintillation spectrometry. The weights of the recipient animals for all studies were not

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[1]Deceased.

statistically different. For the serum clearance study, rats given OA-enriched chylomicrons weighed 283 ± 8.4 g, and those given EPA-enriched chylomicrons weighed 287 ± 5.5 g. Each group comprised eight or nine rats.

Clearance of chylomicrons and redistribution of the labeled EPA or OA among the serum lipoproteins were performed by using similar bolus injections of chylomicrons, prepared as above, into the anesthetized recipient rats. Four rats were used for each time period and each group. Their body weights varied from 262 ± 7.5 g to 276 ± 10.0 g. At either 0, 5, 30, 90 or 240 min, animals were killed by cardiac puncture. Serum was separated by centrifugation (100 × g), and aliquots were subjected to ultracentrifugation to separate major lymph lipoproteins as described by Havel et al. (23). These included chylomicrons (d < 1.006 g/ml, 3 × 10^6 g min), very low density lipoproteins (VLDL, d < 1.006 g/ml, 1 × 10^8 g min), intermediate density lipoproteins (IDL, d = 1.006–1.019 g/ml, 1 × 10^8 g min), low density lipoproteins (LDL, d = 1.1019–1.063 g/ml, 1.3 × 10^8 g min) high density lipoproteins (HDL, d = 1.063–1.21 g/ml, 1.7 × 10^8 g min) and infranatant fraction (d > 1.21 g/ml, 1.7 × 10^8 g min). Aliquots (100–250 µl) of each lipoprotein fraction were taken for liquid scintillation spectrometry. Student's t-test was used to analyze the data.

RESULTS

As reported earlier (22), about three-quarters of the absorbed OA or EPA was recovered as lymph chylomicrons (62.5% for OA and 75.8% for EPA), and the majority of the remainder was recovered with the d < 1.006 g/ml VLDL fraction. Analyses of these chylomicron lipids from donor rat lymph (Table 1) showed that almost 90% of the OA and nearly 87% of the EPA were associated with the chylomicron triglycerides. These distributions are also comparable to those reported earlier (22).

Figure 1 shows the clearance of total radioactivity from serum over 90 min after a single bolus of either OA- or EPA-enriched chylomicrons. During the first 2 min after infusion, the disappearance rates of radioactivity for OA and EPA were similar and represented 44–50% clearance of these lipids from the circulation. At periods of 25 min up to 90 min, there were small but significant differences ($P < 0.05$) between the residual radioactivities in the serum for the EPA and OA groups.

The specific clearance curves for chylomicron radioactivity are summarized in Figure 2. For these studies, four rats were killed at each of the four time periods for each bolus infusion, and chylomicrons were assayed in each serum sample. Again the disappearance rate of chylomicron radioactivity was comparable for the two recipient groups, and except for the 90-min analysis, showed no statistical differences. Thus, approximately 60% of chylomicron radioactivity was cleared from serum by 5 min, 92–95% by 30 min and 96–98% by 90 min, and less than 2% chylomicron radioactivity remained by 240 min.

TABLE 1

Distribution of Radiolabeled Oleic Acid or Eicosapentaenoic Acid among the Lymph Chylomicron Lipids

Administered fatty acid	Chylomicron lipids (% distribution)				
	TG	MG-DG	PL	UFA	CE
Oleic	90.5	1.3	2.0	2.8	3.4
Eicosapentaenoic	86.8	3.0	3.9	2.1	4.2

Treatment of animals, collection of lymph and separation of lymph lipoproteins and lipids are described in Materials and Methods. TG, triglycerides; MG-DG, mono- and diglycerides; PL, phospholipids; UFA, unesterified fatty acids; CE, cholesteryl esters.

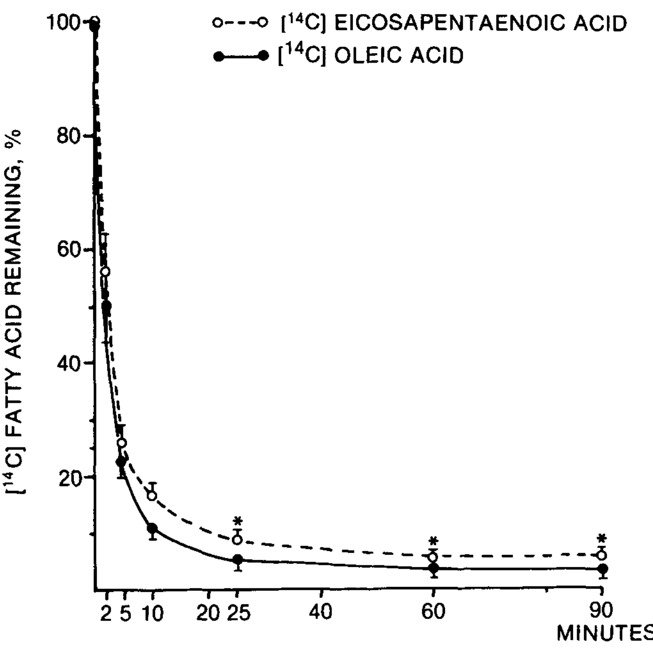

FIG. 1. Clearance of ^{14}C-labeled fatty acids in serum after a single bolus injection of oleic acid- or eicosapentaenoic acid-enriched chylomicrons. Each point is the mean ± S.E. of values from eight or nine rats. *, $p < 0.05$.

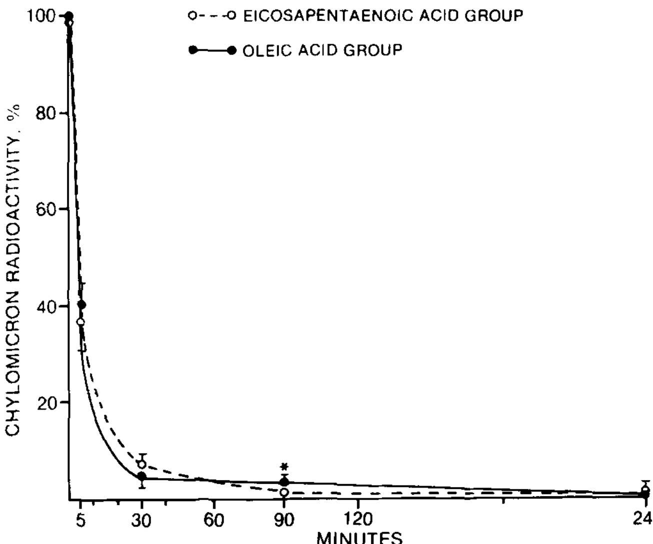

FIG. 2. Chylomicron clearance (% chylomicron remaining) after a single bolus injection of oleic acid- or eicosapentaenoic acid-enriched chylomicrons. Each point is the mean ± S.E. of values from four rats. *, $p < 0.05$.

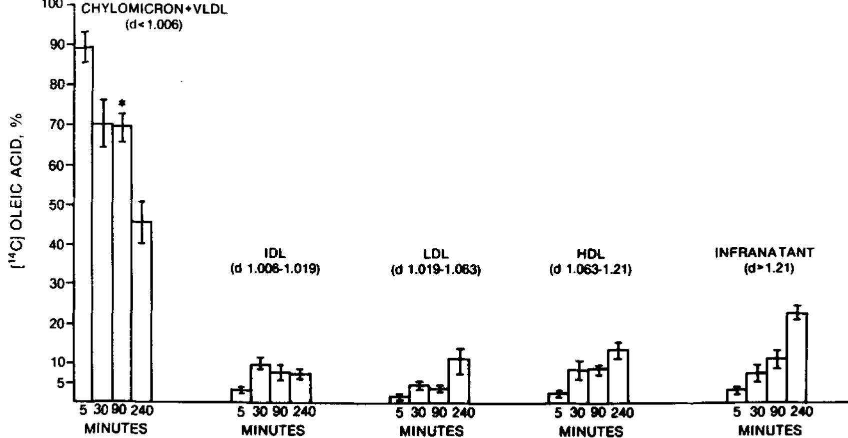

FIG. 3. Redistribution of [14C]oleic acid in serum lipoproteins (%) after a single bolus injection of oleic acid-enriched chylomicrons. Each point is the mean ± S.E. of values from four rats. *, $p < 0.05$.

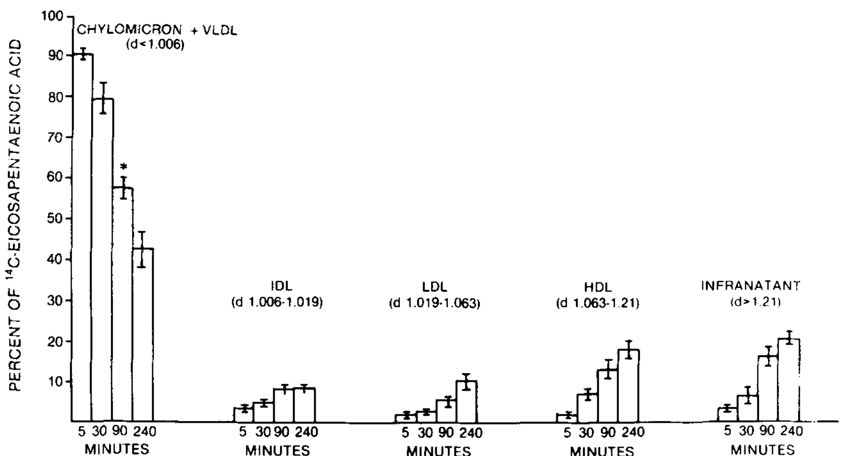

FIG. 4. Redistribution of [14C]eicosapentaenoic acid in serum lipoproteins (%) after a single bolus injection of eicosapentaenoic acid-enriched chylomicrons. Each point is the mean ± S.E. of values from four rats. *, $p < 0.05$.

The distributions of the serum radioactivity among various lipoprotein fractions at periods up to 240 min after infusion are shown in Figures 3 and 4. With OA-enriched chylomicrons (Fig. 3), after 5 min, when total serum radioactivity had decreased to about 60% of that infused, almost 90% was recovered as d < 1.006 g/ml lipoproteins, with almost equal amounts in the remaining lipoprotein and lipoprotein-free serum fractions. By 30 min, there were increases in the percentage of remaining radioactivity in d > 1.006 g/ml lipoproteins. By 240 min, when serum radioactivity represented less than 2% of the injected dose, d > 1.006 g/ml fraction radioactivity had increased to over half (55%) of the total, with the largest increases in the higher density lipoproteins (d > 1.019 g/ml LDL and HDL) and the lipoprotein-free fraction.

Similar temporal redistributions of radioactivity were evident when rats were given EPA-enriched chylomicrons (Fig. 4).

DISCUSSION

The present study was designed to assess whether chylomicron triglycerides enriched in EPA were lipolyzed efficiently by membrane-supported lipoprotein lipase of the capillary vessels and subsequently cleared efficiently from the circulation. This experimental design was based on a variety of earlier reports concerning the poor digestibility of triglycerides containing eicosapentaenoate (18,19) due to steric hindrance by EPA and the suggestion that absorbed salmon oil was cleared more rapidly from plasma after absorption from the intestine (17). We have demonstrated that the unesterified EPA is absorbed as efficiently as oleic or arachidonic acids into the thoracic duct and that the subsequent lymphatic transport and lipoprotein distribution of the absorbed EPA is comparable to that of other fatty acids (22). We also found that the initial peripheral degradation of EPA-enriched chylomicron triglycerides by membrane-supported lipoprotein

lipase in the heart muscle is similar to that with chylomicrons enriched with oleate (20). Our present study demonstrates that the overall clearances of the EPA-enriched and oleate-enriched chylomicrons from the circulation are essentially the same. The small differences observed between the residual radioactivities of the injected fatty acids in the serum at the later time periods (Fig. 1) cannot be ascertained. Further studies are needed to clarify this point.

Unlike intestinal pancreatic lipase, the membrane-supported lipoprotein lipase apparently shows little discrimination in the hydrolysis of triglycerides enriched with either EPA or OA. This was also observed during recirculating heart perfusion in vitro (20). Thus, there is no evidence of steric hindrance, as appears to be the case for pancreatic lipase in hydrolysis of fish oil triglycerides containing EPA (18,19).

The redistribution of labeled fatty acid among serum lipoproteins during chylomicron metabolism was comparable for the two fatty acids. However, the distribution of each fatty acid among the major esterified lipid fractions in each ultracentrifugal fraction was not determined. Based on other studies (24), it is likely that the subsequent metabolism of EPA-enriched chylomicrons results in incorporation of EPA largely into the phospholipid fraction of tissue membranes and lipoproteins, in contrast to the distribution of oleate.

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Thermal and Metal-Catalyzed Decomposition of Methyl Linolenate Hydroperoxides

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Much work has been reported on the volatile oxidative products of fats and their impact on flavor deterioration, cellular damage and the decrease in safety of fat-containing foods. However, relatively little information is available on the mechanism of hydroperoxide decomposition. Pure methyl linolenate hydroperoxides were decomposed thermally at 150 C and catalytically with ferric chloride-ascorbic acid at room temperature. The volatile decomposition products were collected on porous polymer (Tenax) traps and concentrated by gel permeation chromatography. The total volatile products showed significant differences in composition by capillary gas chromatography-mass spectrometry (GC-MS). Thermal decomposition produced much more methyl octanoate (60.1%) and less 2,4-heptadienal (0.5%) than catalytic decomposition (13.2 and 60.8%, respectively). The volatiles from the ferric chloride-ascorbic acid system also contained unique products tentatively identified by GC-MS as isomers of chloromethyl butene. These results may have important implications in evaluating precursors of flavor deterioration in vegetable oils containing linolenate and in understanding better the biological significance of lipid peroxidation.
***Lipids 22,* 322-327 (1987).**

Foods containing polyunsaturated fats and vegetable oils develop flavor problems when subjected to prolonged storage (1), when heated at the elevated temperatures of cooking and frying (2,3) and when exposed to light (4). Despite the considerable information now available on the oxidative deterioration of fats (5-8), the mechanism of this oxidatively derived flavor deterioration is still not well understood. The association of volatile oxidation products with cellular damage (9,10) and with a decrease in safety of fat-containing foods (5) is also not well supported by direct evidence.

The 9- and 13-isomers of linoleic and linolenic acid hydroperoxides prepared by lipoxygenase were previously decomposed in the presence of metals and reducing agents, and the aldehydes formed were identified as the 2,4-dinitrophenyl hydrazone (DNPH) derivatives (11,12). In other studies the isomeric mixtures of hydroperoxides formed by autoxidation and photosensitized oxidation were decomposed thermally, and the volatile products were investigated by gas chromatography-mass spectrometry (GC-MS) (13). This technique permits identification of a wide variety of hydrocarbons, aldehydes, alcohols, esters, aldehyde esters, furans, ketones and lactones. The mechanisms of decomposition of different fatty ester hydroperoxides (13,14) and secondary lipid oxidation products (15,16) were clarified by GC-MS analyses of volatile decomposition products.

Primary and secondary products of lipid oxidation were shown to react with DNA by determining the fluorescence formed in the presence of metals and ascorbic acid (17). The same oxidation products were tested by the Ames reversion assay and found to be weakly mutagenic in *Salmonella typhimurium* strains TA 97 and/or TA 100 (18). In previous work (19), we used GC-MS to study the thermal and acid decomposition products of linoleate and linolenate hydroperoxides and secondary products. In this paper we compare the volatile products obtained from pure methyl linolenate hydroperoxides decomposed thermally at 150 C and catalytically in the presence of ferric chloride and ascorbic acid at room temperature. The volatiles, trapped in a porous polymer (Tenax) column and concentrated by gel permeation chromatography (GPC), were analyzed by capillary GC and GC-MS.

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EXPERIMENTAL PROCEDURES

Preparations. Pure hydroperoxides were prepared by silicic acid column chromatography from methyl linolenate autoxidized at 40 C (20). The purity of the hydroperoxides was checked by thin layer chromatography (TLC) and by peroxide value (6100 me/kg) determination. Analysis of isomers by high pressure liquid chromatography of the hydroxystearate derivatives (21) gave the following composition: 31% 9-, 12% 12-, 12% 13- and 45% 16-hydroperoxides. For the isolation of volatile decomposition products it was essential to remove the antioxidant BHT used as preservative in diethyl ether. Redistilled reagent-grade diethyl ether was chromatographed in 50-ml portions through a short column containing 10 g silicic acid previously activated at 120 C. The resulting ether was stored at 0 C and checked for peroxide iodometrically before use.

Tenax (Anspec Co., Ann Arbor, Michigan) used to trap volatiles was activated by washing 3-g portions placed in a chromatographic column with 50 ml distilled and chromatographed diethyl ether. The column was then purged with dry nitrogen for 30 min at room temperature until the ether was removed and at 150 C overnight.

Decomposition. Methyl linolenate hydroperoxides were decomposed either thermally at 150 C or catalytically with ferric chloride and ascorbic acid at room temperature in a reaction flask attached to a trap containing 500 mg activated Tenax porous polymer. Reactions were continued until the hydroperoxides were almost completely decomposed as indicated by TLC (20).

For thermal decomposition, 500 mg of methyl linolenate hydroperoxides was purged with air, which was purified through a drying tube (sodium sulfate) and a short Tenax trap, and heated at 150 C for 30 min. After cooling to room temperature, the Tenax trap was weighed and then washed with 5 ml chromatographed diethyl ether. The amount of volatiles trapped was determined by reweighing the Tenax after removing the solvent and drying. An aliquot of the Tenax ether eluate was analyzed directly by capillary GC. The decomposition mixture was dissolved in methylene chloride and separated by GPC. The low molecular weight fraction was made up to 5.0 ml with methylene chloride, and the weight of decomposition

products was determined from an aliquot by gently removing the solvent at 0 C.

Conditions for the catalytic decomposition with ferric chloride and ascorbic acid were similar to those used previously for fluorescence formation in the presence of DNA (17). Methyl linolenate hydroperoxides (500 mg) were stirred in 1.8 ml buffer solution at pH 6.1 (100 ml 0.1 M KH_2PO_4 and 14 ml 0.1 M NaOH) containing 0.06 ml Tween-20 and 1.2 ml ascorbic acid (2.76 g per 25 ml water). The reaction flask was attached to a Tenax trap. The iron catalyst (1.3 ml of a solution containing 3.83 g $FeCl_3$ in 25 ml water) was then added, and the reaction mixture was purged with purified air and stirred magnetically for 4 hr at room temperature. The Tenax trap was eluted with diethyl ether as above. The residual emulsion was transferred with water and extracted with chromatographed diethyl ether. The ether extract was concentrated by gently sweeping with nitrogen, and the product was separated by GPC.

GPC. A DuPont high performance liquid chromatograph (DuPont Instruments, Wilmington, Delaware) was used with two glass columns (9 × 1000 mm) connected in series and packed with Bio-Beads S-X2 (Bio-Rad Labs, Richmond, California). Methylene chloride was used as mobile phase at a flow rate of 0.5-1 ml/min and pressure of 16-19 bars. Samples of 30 mg dissolved in methylene chloride were injected, and the separation was followed with refractive index (Waters Assoc., Milford, Massachusetts) and ultraviolet detectors (DuPont at 274 nm). Molecular weights of recovered fractions were determined by vapor pressure osmometry using benzene as solvent.

GLC. Volatile decomposition products recovered from the Tenax and GPC eluates were analyzed by GLC with a Perkin-Elmer instrument (Model Sigma 300, Norwalk, Connecticut), equipped with a capillary column (DB 1701, J & W Scientific Co., Rancho Cordova, California) cooled to −20 C with liquid nitrogen. Column temperature was programmed to 260 C at 5 C per min with a final hold of 10 min. The integrated relative peak areas were calculated as weight percent. Methyl hexanoate was used as internal standard. Volatile decomposition products were identified like before (13,22) by matching mass spectra with those of our reference library and by confirming GC retention data with known reference compounds.

RESULTS

Previous studies on the aldehyde decomposition products of oxidized fats were based mainly on analyses of suitable DNPH derivatives (23), which are difficult to prepare and to regenerate from polyunsaturated carbonyl compounds. GC and GC-MS methodologies have provided a significant improvement by affording direct analyses of volatile aldehyde and nonaldehyde products formed by lipid oxidation. In the present study, improved techniques, such as Tenax trapping and GPC, were used together with capillary GC and GC-MS to investigate the volatile oxidation products generated from linolenate hydroperoxides at room temperature.

The porous polymer Tenax (poly[2,6-diphenyl-p-phenylene oxide]) is considered one of the best adsorbents for concentration of organic volatile compounds, with many applications in flavor, atmospheric pollution and headspace analyses of biochemical samples (24,25). In this work, volatile products formed during the decomposition of linolenate hydroperoxide were trapped in a Tenax column. While the recovery of hydrocarbons and simple alcohols and ethers was reported to be in the 75-90% range, some of the simple alcohols were retained poorly with Tenax (25).

GPC has been used to separate and analyze high molecular weight products in heated fats (26) and oxidized methyl linoleate (27) and linolenate (28). In this study, we used a similar GPC system to separate low molecular weight volatile compounds from decomposed methyl linolenate hydroperoxides. The GPC system used consisted of polystyrene divinyl benzene beads and methylene chloride as solvent. The separation was followed by refractive index and UV detection (Fig. 1). In the top chromatograms, obtained with the sample decomposed with ferric chloride-ascorbic acid, the refractive index curve shows a major peak due to oligomer with a molecular weight of 625, representing 73% (by weight) of the total. The UV curve shows greater absorption at 274 nm in the monomer than in the oligomer peak. The third peak is due to low molecular weight volatile decomposition products, which are not detected by refractive index. The

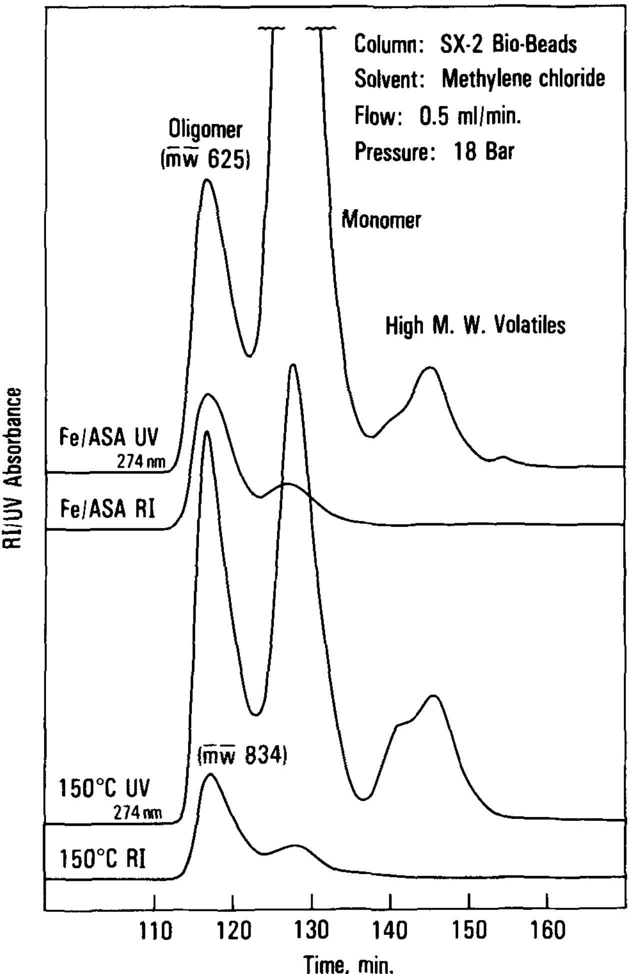

FIG. 1. Gel permeation chromatography of decomposition products of methyl linolenate hydroperoxides. Top curves: Fe/ASA, ferric chloride-ascorbic acid; bottom curves, thermal at 150 C. (UV, ultraviolet detection at 274 nm; RI, refractive index detection.)

bottom chromatograms, obtained with the hydroperoxides decomposed at 150 C, show the same relationship. The refractive index curve shows a major oligomer peak, representing 62% by weight, with a molecular weight of 834. The UV curve shows a significant peak after the monomer peak, due to the high molecular weight volatile decomposition products.

The monomeric GPC-2 fractions were examined by TLC and spectrophotometry. The absence of unreacted hydroperoxides was confirmed by TLC (20). The monomers from samples decomposed with ferric chloride–ascorbic acid gave more intense UV absorption at 275 nm, due to conjugated keto dienes (29), than the monomers from thermally decomposed hydroperoxides. Weak infrared bands, due to conjugated carbonyls, were also obtained at 1639 cm^{-1} with the thermally decomposed sample, and at 1633 and 1687 cm^{-1} with the catalytically decomposed sample. Although TLC indicated the absence of hydroperoxides, weak UV absorption obtained at 231 nm may indicate the presence of epoxy or epidioxy compounds with diene conjugation. Further structural studies on the oligomer and monomer GPC fractions (GPC-1 and GPC-2) are reported elsewhere (28).

The relative weight compositions of the volatiles recovered by Tenax trapping and GPC are summarized in Table 1. Total volatiles, including the Tenax eluate and GPC low molecular weight fraction, represented 7.43% in the linolenate hydroperoxides decomposed at 150 C and only 2.1% in the sample decomposed with ferric chloride and ascorbic acid. Although these volatile products represent a small portion of the total decomposition materials, they probably have an important impact on flavor and biological effects of lipid oxidation (14).

Capillary GC analyses showed that the aldehydes and other volatile compounds recovered on the Tenax trap were of lower molecular weight than the volatiles obtained by GPC. Tenax volatiles produced by thermal decomposition included significant amounts of propanal, 1-penten-3-ol, 2-pentenal, 2,4-heptadienal and methyl octanoate and small amounts of butanal, ethyl furan, 2-butenal, 1-penten-3-one and methyl heptanoate (Table 2). On the other hand, the Tenax volatiles produced by decomposition with ferric chloride and ascorbic acid included significantly higher relative concentrations of propanal, butanal and 2,4-heptadienal, much less methyl octanoate and no ethyl furan, 1-penten-3-one, 1-penten-3-ol or 2-pentenal.

In addition, GC-MS indicated major amounts of chloromethyl butenes in the Tenax volatiles from the sample decomposed with ferric chloride–ascorbic acid. The mass spectra obtained for these unusual components in the chromatograms were in good agreement with library spectra for 1-, 2- or 3-(chloromethyl)1-butene, with major masses at m/z 41, 53, 55, 69, 89 and 104, due to the molecular ion for the elemental formula C_5H_9Cl. Although the matches were good, this identification is only tentative because the authentic compounds were not available. The origin of these chlorinated compounds is unknown.

The volatiles separated from the reaction mixtures by GPC were of higher molecular weight than the Tenax volatiles. The GPC volatiles produced by thermal decomposition included a significant amount of methyl octanoate, smaller amounts of 2,4-heptadienal, 2,4,7-decatrienal and methyl 9-oxo-nonanoate and minor amounts of methyl heptanoate and methyl 8-oxo-octanoate (Table 2). In comparison, the GPC volatiles produced by decomposition with ferric chloride–ascorbic acid included much larger amounts of 2,4-heptadienal and smaller amounts of methyl octanoate and 2,4,7-decatrienal.

The weight percent compositions of both Tenax and GPC fractions were used to calculate the total volatile composition. The total volatiles recovered after thermal decomposition included major amounts of methyl octanoate and smaller amounts of 2,4-heptadienal, 2,4,7-decatrienal and methyl 9-oxo-nonanoate (Table 2). On the other hand, the total volatiles recovered after decomposition with ferric chloride–ascorbic acid included major amounts of 2,4-heptadienal and smaller amounts of methyl octanoate and 9-oxo-nonanoate.

DISCUSSION

Working with autoxidized methyl linolenate, Kimoto and Gaddis (30) found no difference in the distribution of monocarbonyls, identified as DNPH derivatives, after

TABLE 1

Relative Weight Composition of Volatile Fractions from the Decomposition of Methyl Linolenate Hydroperoxides

		Thermal (150 C)		Fe-ascorbate	
Fractions[a]	Oxidation products	Wt (mg)	Rel %	Wt (mg)	Rel %
Tenax	Low mol wt volatiles	3.5	0.7	1.0	0.2
GPC-1	Oligomer	320.3	61.6	368.4	72.7
GPC-2	Monomer	161.2	31.0	128.0	25.3
GPC-3	High mol wt volatiles	35.1	6.7	9.6	1.9
Total		520.0	100.0	507.0	100.0
Total volatiles[b]		38.6	7.4	10.6	2.1

[a]Tenax fractions include low mol wt volatiles (propanal, butanal, ethyl furan, 2-butenal, 1-penten-3-one, 1-pentene-3-ol, 2-pentenal, methyl heptanoate, 2,4-heptadienal and methyl octanoate; see Table 2). GPC-3 fractions (see Fig. 1) include higher mol wt volatiles (methyl heptanoate, 2,4-heptadienal, methyl octanoate, 2,4,7-decatrienal and methyl 9-oxo-nonanoate; see Table 2).

[b]Total volatiles = Tenax + GPC-3 fractions.

TABLE 2

Volatile Decomposition Products of Methyl Linolenate Hydroperoxides (Relative Weight Percentage[a])

	Thermal (150 C)			Fe-ascorbate		
Volatile products	Tenax fractions	GPC-3 fractions	Total volatiles[c]	Tenax[b] fractions	GPC-3 fractions	Total volatiles[c]
Propanal	10.6	0.0	1.0	24.7	0.0	2.3
Butanal	1.4	0.0	0.1	18.8	0.0	1.8
Et furan	3.4	0.0	0.3	0.0	0.0	0.0
2-Butenal	5.4	0.0	0.5	8.6	0.0	0.8
1-Penten-3-one[d]	6.8	0.0	0.6	0.0	0.0	0.0
1-Penten-3-ol	17.4	0.0	1.6	0.0	0.0	0.0
2-Pentenal	14.9	0.0	1.5	0.0	0.0	0.0
Me heptanoate	2.3	1.0	1.1	2.7	2.2	2.2
2,4-Heptadienal	9.6	10.7	10.5	41.0	62.9	60.8
Me octanoate	28.2	63.5	60.1	4.2	14.2	13.2
2,4,7-Decatrienal	0.0	13.2	12.0	0.0	7.4	6.7
Me 8-oxo-octanoate	0.0	3.0	2.7	0.0	2.2	2.0
Me 9-oxo-nonanoate	0.0	8.9	8.1	0.0	11.2	10.2
Total volatiles	91.1	89.4	100.0	30.0	93.6	100.0
Unknowns	8.9	10.6	—	70.0[b]	6.4	—

[a]Based on concentration of methyl hexanoate used as internal standard.

[b]Tenax volatiles include major amounts of chloromethyl butenes tentatively identified by GC-MS (see text).

[c]Tenax + GPC-3 fractions weighted according to percentage of each fraction (see Table 1).

[d]Includes formic acid.

decomposition by heat (165 C) and by ferrous chloride, cupric stearate and hematin. They observed major amounts of propanal and 2,4-heptadienal and smaller amounts of 2-pentenal and what was believed to be trienals. Later, Grosch (12) also identified the monocarbonyls (as DNPH derivatives) formed from pure linolenate hydroperoxides decomposed with trace metals in the presence of ascorbic acid. He reported major amounts of propanal and 2-hexenal from the 9-hydroperoxide isomer and 2-pentenal and 2-hexenal from the 13-hydroperoxide isomer.

When we analyzed by GC-MS the thermal decomposition products from the mixture of 9-, 12-, 13- and 16-hydroperoxides formed by autoxidation of linolenate (13), the major volatiles included ethane, 2,4-heptadienal, methyl octanoate, decatrienal and methyl 9-oxo-nonanoate. The mechanism advanced to explain the major volatile decomposition products (14) involves homolytic cleavage on either side of the hydroperoxide groups (Fig. 2). Significant differences were also observed between thermal and acid decomposition products from monohydroperoxides and secondary oxidation products (19). The differences in volatile decomposition patterns were explained by assuming that thermal decomposition proceeds by homolytic β-scission on both sides of the hydroperoxide group, whereas acid decomposition involves selective heterolytic splitting between the hydroperoxide group and the allylic double bond. On theoretical grounds, a "mixed" homolytic-heterolytic type mechanism was also invoked for the thermal decomposition of hydroperoxides (19).

In the present work, significant qualitative and quantitative differences were found in the volatiles products obtained by decomposing linolenate hydroperoxides by

FIG. 2. Main cleavage mechanism recognized for linolenate hydroperoxides. Percentages given on the right are the weight composition found for the hydroperoxides of methyl linolenate. R = $-(CH_2)_7-COOCH_3$.

TABLE 3

Origin of Major Volatile Products from Decomposition of Methyl Linolenate Hydroperoxides

Major volatiles	Thermal (150 C) (Rel %)	Fe-ascorbate (Rel %)	Origin (Ln-OOH[a])	Cleavage type[b]
2,4-Heptadienal	10.5	60.8	12-OOH	Homolytic
Methyl octanoate	60.1	13.2	9-OOH	Homolytic
2,4,7-Decatrienal	12.0	6.7	9-OOH	Homolytic
Methyl 9-oxo-nonanoate	8.1	10.2	9-OOH	Heterolytic
1-Penten-3-ol	1.6	0.0	13-OOH	Homolytic
2-Pentenal	1.5	0.0	13-OOH	Heterolytic
Propanal	1.0	2.3	16-OOH	Heterolytic

[a]Linolenate hydroperoxide isomers, based on scheme in Fig. 2.

[b]Based on scheme in ref. 19: homolytic cleavage occurs on both sides of the hydroperoxide group; heterolytic cleavage occurs selectively on one side between the hydroperoxide group and the allylic double bond.

heating at 150 C or by treating with ferric chloride-ascorbic acid at room temperature. Thermal decomposition produced more volatiles (7.4%), which were dominated by methyl octanoate. Decomposition with ferric chloride-ascorbic acid produced less volatiles (2.1%), which were dominated by 2,4-heptadienal. Table 3 summarizes the major volatiles, their probable hydroperoxide origin and the cleavage types, which account for their formation. Although 2,4-heptadienal is the major decomposition product observed with ferric chloride-ascorbic acid, it is derived from the 12-hydroperoxide isomer, which constitutes only 12% of the hydroperoxides in autoxidized methyl linolenate (Fig. 2) (6). On the other hand, the major thermal decomposition product, methyl octanoate, originates from the 9-hydroperoxide isomer, which constitutes 31% of the linolenate hydroperoxides. 2,4,7-Decatrienal and methyl 9-oxononanoate, formed in smaller amounts under both thermal and metal-catalyzed decomposition, also originate from the 9-hydroperoxide isomer.

The differences in volatile profiles obtained between decomposition by heat and by ferric chloride-ascorbic acid cannot be attributed to different cleavage mechanisms because the main volatile products obtained under both conditions can be formed by homolytic cleavage of either the 9- or 12-hydroperoxide isomers (Fig. 2). However, the minor volatile compounds including butanal, ethyl furan, penten-3-one, 1-penten-3-ol, 2-pentenal, methyl octanoate and 8-oxo-octanoate, cannot be explained by the homolytic cleavage mechanism in Figure 2. A more complex mixture of minor compounds was produced by heat than by decomposition with ferric chloride-ascorbic acid. Unique thermal decomposition products included ethyl furan, penten-3-one, 1-penten-3-ol and 2-pentenal (Table 2). 1-Penten-3-ol can be explained by the same mechanism suggested previously for 1-octen-3-ol (14), involving the reaction of a hydroxyl radical with the 2-pentene radical formed by homolytic cleavage of the 13-linolenate hydroperoxide. However, the origin of the other volatiles is unclear. The differences in the volatile profiles observed in this study suggest that heat treatments of oils and metal contamination may have different effects on the flavor deterioration of oils containing linolenate.

Other significant decomposition products of linolenate hydroperoxides include oligomers and monomeric secondary oxidation products. The oligomer fraction isolated by GPC (Fig. 1) was found to be a rich source of volatiles (28). Like monohydroperoxides and secondary oxidation products, these high molecular weight volatile precursors may have an important impact on flavor deterioration in unsaturated fats.

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Efficacy of Linoleic Acid Administered Rectally as Monoglyceride

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The potential of the rectal route for administration of essential fatty acids (EFA) as monoglyceride (MG) was investigated. EFA-deficient rats were supplemented with 14 mg linoleic acid/day for 3 days. Supplementation was either by oral administration as corn oil, orally as corn oil-derived MG or rectally as MG. The patterns of polyunsaturated fatty acids (PUFA) in liver and serum lipids, characteristic of EFA deficiency, were altered in the direction of normalcy in similar magnitude by all modes of supplementation, indicating that the rectal route may be useful for administration of EFA. The amounts of phospholipids (PL) and free fatty acids (FFA) in liver changed by all modes of administration. The magnitude of change of total PL and of FFA in liver depended upon the chemical form in which linoleic acid was administered and the route of administration, indicating that these factors affect lipid metabolism.
Lipids 22, 328–332 (1987).

Patients with partial loss of the small intestine cannot absorb orally fed fats, and eventually develop EFA deficiency (1). Intravenous fat alimentation may provoke signs of intolerance (2), thus limiting this treatment for EFA deficiency (1,3), and alternative routes for providing EFA may be of value. Dermal application of oils (4–6) or of fatty acid mixtures (7) rich in EFA have been tried, with variable results. In humans (8), rabbits (9) and dogs (10), medium chain triglycerides (TG) are taken up when administered rectally. In rabbits, saturated long chain TG administered rectally are absorbed, but unsaturated long chain TG are not (10). In man, small doses of linoleic acid administered in suppositories as sodium linoleate (11) are absorbed. MG have physico-chemical properties considered favorable for rectal uptake (12) and have been useful in introducing fat orally in instances of impaired digestion and transport (13,14). The purpose of this study was to explore the potential of the rectal route for the administration of linoleic acid MG to relieve the biochemical indicators of EFA deficiency, and changes in lipid levels were measured quantitatively. The short-term effects of rectal administration of a suboptimal dose of linoleic acid MG, equal to one-third of the estimated minimum daily requirement, were measured. The results were compared with those obtained after oral administration of the same amount of linoleic acid either as MG or TG.

MATERIALS AND METHODS

Experimental animals. EFA deficiency was induced by feeding male Sprague-Dawley weanling rats a fat-free semipurified diet for 4 weeks (15). The diet consisted of 25.5% vitamin-free casein, 65% sucrose, 4% cellulose (alphacel, NBCo Biochemicals, Division of ICN Biochemicals, Cleveland, Ohio), 3.5% Williams-Briggs salt mix (ICN) and vitamins (3). The diet was provided ad libitum, and the animals had free access to water. Male Sprague-Dawley rats fed from weaning the same diet with corn oil added (2.7:100) served as normal controls on an adequate intake of EFA. Rats were kept individually in an environmentally controlled laboratory animal facility (50–60% relative humidity, 22–24 C, 12-hr light/dark cycle, laminar flow ventilation) in cages with wire floors to minimize coprophagy. The average weight of the EFA-deficient rats was 185 g at age 7 wk. An isomeric equilibrium mixture of MG prepared from corn oil was obtained from Eastman Kodak (Myverol® type 18-92; Rochester, New York). Diet containing MG and diet containing corn oil were prepared by mixing 2.7 g MG or 2.5 g corn oil, respectively, dissolved in 300 ml acetone and 100 g fat-free diet and evaporating the acetone under vacuum at a temperature not exceeding 35 C. These diets, which contained 14 mg linoleic acid/g, were stored under N_2 at 4 C and given daily for 3 days. The fatty acid compositions of corn oil and corn oil-derived MG were, respectively, 16:0, 6.2 and 6.6%; 18:0, 4.6 and 4.2%; 18:1ω9, 16.4 and 16.4%; 18:2ω6, 63.4 and 65.6%; 18:3ω3, 0.1 and 0.1%; 20:3ω3, 0.4 and 0.3%.

Experimental protocol. The EFA-deficient rats were divided into four groups of five animals each. The first group continued to receive the fat-free diet ad libitum. The second group received the fat-free diet ad libitum, but each evening for three days, 27 mg of the linoleic acid-rich MG containing 14 mg linoleic acid was given rectally. The MG was molten (melting point 41 C) and was delivered with a Gilson pipet with a prewarmed plastic disposable tip from which the point was cut. The plastic tip was inserted about 1.5 cm into the rectum. Usually, the rats' being handled provoked defecation, after which the MG was delivered. No leakage of MG was observed, and the treatment did not result in diarrhea. The third and fourth groups received the fat-free diet ad libitum only during the day. Each day for three days this food was removed in the evening and replaced by 1 g of diet containing MG or corn oil for the oral MG and oral TG groups, respectively. In this way, all the rats ate the predetermined amount of linoleic acid (14 mg/day). After three days' administration of linoleic acid to the second, third and fourth groups, all rats were killed by decapitation and their blood and livers were collected.

Liver and serum lipids were extracted with chloroform/methanol (2:1, v/v) as described previously (16,17). The extract was dried under vacuum, the amount of total lipid was determined gravimetrically and the residue was redissolved in chloroform (1 ml/10 mg of lipid). One ml of chloroform solution containing 1 mg of diheptadecanoyl *sn*-3 phosphatidylcholine (Sigma Chemical, St. Louis, Missouri), 80 μg pentadecanoic acid, 750 μg triheptadecanoate and 300 μg cholesteryl heptadecanoate (all from Nu-Chek Prep, Elysian, Minnesota) was added to 1 ml of the liver lipid extract. Liver PL, FFA, TG and cholesteryl esters (CE), with their respective internal standards, were isolated by thin layer chromatography (15) and converted into methyl esters by treatment with 14% BF_3 in methanol for 90 min at 75 C. The same lipid classes from serum were separated by silicic acid-impregnated

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paper (ITLC paper, Gelman Instruments Co., Ann Arbor, Michigan) (16) and converted into methyl esters by the same method. The methyl esters were analyzed by gas chromatography using a 50 m × 0.2 mm i.d. wall-coated open tubular capillary column loaded with FFAP (Scientific Glass Engineering, Austin, Texas) with an He column flow of 0.45 ml/min and a split ratio of 1:70, temperature-programmed from 190-220 C at 2 C/min and held for 18 min at 220 C. Peak identification was made by comparison with known methyl ester standards (Nu-Chek-Prep). The internal standards enabled quantification of the fatty acids of liver PL, FFA, TG and CE. The analytical data were entered into a PDP-12 computer (Digital Equipment Corp., Maynard, Massachusetts) and appropriate FOCAL programs were used (16) to calculate the parameters, which have proven useful to evaluate changes in the pattern of fatty acids (3). Significances between different groups were calculated using Student's t-test for comparison of the means. For the rats on the fat-free diet that were or were not given MG rectally, 50 μl of the total liver extract was fractionated to estimate the amount of total PL and their subfractions by phosphorus content (18).

RESULTS

The fatty acid compositions of the liver PL of rats of the four groups were measured, and the percentages and standard errors of the means of fatty acids in liver PL are included in Table 1. The rats on the fat-free diet had a much higher level of 20:3ω9 and much lower levels of 18:2ω6 and 20:4ω6 in their liver PL than did the rats on the EFA-adequate diet (results not shown), indicating EFA deficiency in the former group. Similar analyses of liver CE, TG and FFA and of serum PL, CE, TG and FFA confirmed EFA deficiency in the fat-free group (data not shown).

Table 1 shows that each mode of linoleic acid administration significantly affected the fatty acid composition of liver PL, partially correcting the EFA deficiency. The data show that rectal linoleic acid administration as MG was equivalent to supplementation by mouth either as MG or TG, as judged by its effect on the fatty acid composition of liver PL. Comparisons of the fatty acid compositions of liver FFA, TG and CE led to the same conclusion. For almost all parameters measured (Tables 1 and 2), the effect of linoleic acid administration, either rectally as MG or orally as MG or TG, was qualitatively the same on liver lipid fatty acid compositions. Quantitatively, differences between oral or rectal administration were minor. In liver, the rectal administration of linoleic acid-rich MG for three days decreased the percentage of 20:3ω9 in all lipid classes (Tables 1 and 2). This decrease was significant in all lipid classes except CE, in which 20:3ω9 was a minor component (1.8% in the fat-free group). Linoleic acid-rich MG administered rectally significantly increased the percentage of 18:2ω6 in all lipids (Tables 1 and 2) except the TG, in which 18:2ω6 was low even in the EFA-adequate group (2.6%).

In the rectally supplemented group, the amount of PL in the liver measured by its fatty acid content (Table 3) or by its phosphorus content (190 ± 96 vs 310 ± 93 μmol/liver; $p < 0.025$) was significantly lower than in the nonsupplemented group. The amount of FFA was significantly higher in the rectally supplemented group than in the nonsupplemented group (Table 3). Liver weights themselves were not significantly different. Effects of oral supplementation on the amount of PL and FFA in liver were in the same direction but were less pronounced than the effects of rectal supplementation (Table 3). Because the amount of phospholipid in EFA-deficient rat liver was lowered by supplementation with linoleate, the amount of arachidonate decreased in the PL

TABLE 1

Fatty Acid Composition[a] of Liver Phospholipids of Rats on a Fat-Free Diet With or Without Linoleic Acid Supplementation

	Linoleic acid supplementation				Significance expressed as p values[b]			
Parameter	None (1)	Rectal MG (2)	Oral MG (3)	Oral TG (4)	2 vs 1	3 vs 1	4 vs 1	2 vs 4
14:0	0.2 ± 0.15	0.2 ± 0.01	0.1 ± 0.01	0.1 ± 0.02				
16:0	14.9 ± 0.30	15.5 ± 0.29	15.1 ± 0.22	15.9 ± 0.38				
16:1ω7	3.9 ± 0.16	4.2 ± 0.35	4.5 ± 0.27	4.3 ± 0.40				
18:0 B[c]	0.2 ± 0.01	0.3 ± 0.04	0.3 ± 0.03	0.3 ± 0.04	<0.001			
18:0	22.8 ± 0.39	20.0 ± 0.54	20.5 ± 0.26	19.3 ± 1.14	<0.01	<0.001	<0.05	
18:1ω9	12.5 ± 0.57	13.9 ± 0.88	15.0 ± 0.50	13.2 ± 0.97		<0.01		
18:1ω7	4.0 ± 0.16	5.2 ± 0.46	5.5 ± 0.35	5.9 ± 0.26	<0.05	<0.01	<0.001	
18:2ω6	1.4 ± 0.31	3.5 ± 0.46	2.6 ± 0.06	3.4 ± 0.19	<0.01	<0.01	<0.001	
18:3ω6	0.4 ± 0.10	0.3 ± 0.03	0.4 ± 0.06	0.3 ± 0.05				
20:3ω9	17.8 ± 0.58	13.6 ± 1.17	16.0 ± 0.75	14.8 ± 0.48	<0.01		<0.01	
20:3ω6	0.4 ± 0.06	0.9 ± 0.08	0.8 ± 0.05	0.9 ± 0.11	<0.001	<0.001	<0.01	
20:4ω6	11.8 ± 0.36	12.3 ± 1.27	10.4 ± 0.81	11.9 ± 0.75				
22:5ω6	1.6 ± 0.08	1.9 ± 0.31	1.4 ± 0.26	1.7 ± 0.08				
22:6ω3	2.3 ± 0.07	2.8 ± 0.36	2.1 ± 0.14	2.3 ± 0.19			<0.05	<0.01
24:1	0.2 ± 0.02	0.3 ± 0.03	0.2 ± 0.02	0.2 ± 0.28				

MG, monoglyceride; TG, triglyceride.

[a]Weight percentage ± standard error of the mean.

[b]Expressed as p values. No significant differences were observed in comparisons of 2 vs 3 or 3 vs 4.

[c]Branched chain 18:0.

TABLE 2

Selected[a] Fatty Acids in Liver FFA, TG and CE of Rats on a Fat-Free Diet, Supplemented or Not With Linoleic Acid

Lipid class/ fatty acid group	Linoleic acid supplementation				Significance expressed as p values		
	Fat-free (1)	Rectal MG (2)	Oral MG (3)	Oral TG (4)	2 vs 1	2 vs 3	2 vs 4
FFA							
17:0	0.6 ± 0.09	0.2 ± 0.06	0.2 ± 0.01	0.4 ± 0.03	<0.01	<0.001	<0.05
18:0	11.3 ± 0.40	8.8 ± 0.56	6.2 ± 0.39	7.6 ± 1.19	<0.01	NS[b]	NS
18:1ω7	1.1 ± 0.04	0.7 ± 0.03	0.7 ± 0.05	1.0 ± 0.08	<0.001	NS	<0.01
18:2ω6	1.3 ± 0.17	2.4 ± 0.23	1.8 ± 0.13	2.2 ± 0.25	<0.01	<0.05	NS
18:3ω6	0.5 ± 0.06	0.3 ± 0.02	0.3 ± 0.04	0.3 ± 0.06	<0.01	NS	NS
20:3ω9	8.9 ± 0.59	5.6 ± 0.36	4.4 ± 0.59	4.8 ± 0.53	<0.001	<0.05	NS
22:4ω6	0.5 ± 0.04	0.3 ± 0.04	0.2 ± 0.02	0.3 ± 0.03	<0.01	NS	NS
TG							
14:0	0.8 ± 0.04	0.3 ± 0.11	0.5 ± 0.04	0.6 ± 0.06	<0.01	NS	NS
16:0	28.1 ± 0.91	37.1 ± 2.21	33.5 ± 2.61	31.1 ± 1.28	<0.01	NS	<0.05
16:1	8.7 ± 0.12	6.3 ± 0.44	8.4 ± 0.80	8.6 ± 0.66	<0.001	<0.05	<0.05
18:1ω7	0.7 ± 0.05	0.2 ± 0.12	0.4 ± 0.06	0.5 ± 0.04	<0.01	NS	<0.05
20:3ω9	1.9 ± 0.25	0.3 ± 0.18	1.0 ± 0.79	1.1 ± 0.52	<0.01	NS	NS
20:4ω6	0.5 ± 0.08	0.1 ± 0.02	0.6 ± 0.54	0.5 ± 0.35	<0.01	NS	NS
CE							
18:1ω9	63.2 ± 1.03	58.1 ± 1.77	63.3 ± 1.46	60.9 ± 1.23	<0.05	<0.05	NS
18:1ω7	1.2 ± 0.06	0.9 ± 0.05	1.0 ± 0.14	1.1 ± 0.12	<0.01	NS	NS
18:2ω6	0.2 ± 0.05	1.0 ± 0.19	0.7 ± 0.19	0.9 ± 0.20	<0.001	NS	NS

FFA, free fatty acid; TG, triglyceride; CE, cholesteryl ester; MG, monoglyceride.

[a]Only those fatty acids are given (in percentage ± standard error of the mean) where there is a significant difference between fat-free and rectal MG-supplemented groups.

[b]NS, not significant at the 0.05 level.

TABLE 3

Effect of Mode of Administration of Linoleic Acid to EFA-Deficient Rats on Fatty Acids Indicative of EFA Status in Phospholipids and in Free Fatty Acids of Liver[a]

	Fat-free	Linoleic acid administration		
		Rectal MG[b]	Oral MG[b,c]	Oral TG[b]
Liver phospholipids				
18:2ω6	8.3 ± 4.0	16.1 ± 3***	13.5 ± 1.0***	18.8 ± 1.0***
20:3ω9	102.0 ± 7.0	60.0 ± 8***	94.0 ± 8.0†	76.0 ± 3.0**
20:4ω6	68.0 ± 6.0	53.0 ± 6*	59.0 ± 6.0	61.0 ± 3.0
Total fatty acids in PL	613.0 ± 30.0	464.0 ± 17***	528.0 ± 39.0†	557.0 ± 29.0
Liver free fatty acids				
18:2ω6	0.6 ± 1.2	6.9 ± 2***	1.3 ± 0.9†	1.4 ± 0.3
20:3ω9	4.2 ± 1.2	15.0 ± 3**	6.0 ± 1.4†	2.9 ± 0.5
20:4ω6	3.1 ± 1.1	18.0 ± 3**	4.4 ± 1.3	2.5 ± 0.5
Total fatty acids in FFA	50.0 ± 11.0	286.0 ± 46***	71.0 ± 14.0†	65.0 ± 13.0

EFA, essential fatty acids; MG, monoglycerides; TG, triglycerides; PL, phospholipids; FFA, free fatty acids.

[a]Values are μmol/liver ± standard error of the mean.

[b]Significance of difference from fat-free groups is indicated by ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

[c]Significance of difference from rectal MG group is indicated by †, $p < 0.001$.

fraction, although its proportion did not change. There was also a decrease in the amount of 20:3ω9, but an increase in the amount of 18:2ω6. The decrease in the amount of PL was accompanied by an increase in the amount of FFA, which was most pronounced in the rectally supplemented group. As a consequence of the elevated FFA, the reduced percentage of 20:3ω9 was an increased amount.

The reduction in the amount of PL induced by rectal administration of linoleic acid-rich MG was due to decreases in cardiolipin (50% of pretreatment concentration), phosphatidylcholine (55%), phosphatidylethanolamine (69%), phosphatidylserine (67%), sphingomyelin (73%) and phosphatidylinositol (77%) as measured by their phosphorus contents. Lysophospholipid content (104%) was unchanged.

The three modes of administering linoleate each affected the fatty acid compositions of the serum lipid classes. Within each lipid class, the changes induced by linoleate supplementation were generally in the same direction, but differed somewhat in magnitude (results not shown).

DISCUSSION

The MG derivative was chosen for rectal administration of linoleic acid because it is nonionic, fat-soluble yet quite polar, and its physical properties are considered favorable for rectal uptake (12). MG form very viscous nonoily phases in contact with water (19), which mediates against leakage and rapid expulsion from the rectum. The important issue was to measure whether rectal absorption of linoleic acid occurred when administered as MG; this effect was measurable after a very short term of supplementation. A logical control is administration of linoleic acid as TG by mouth, because it is the natural route for EFA supplementation and is thoroughly documented in the EFA-deficient rat. Most of the voluminous literature on nutritional effects of EFA describes studies in which EFA were provided in the form of TG; this literature has been reviewed (3,20,21,22). Because unsaturated TG were reported not to be absorbed from the rectum (10), this control was not included in the study. A control group given oral MG was included to confirm the efficiency of the MG utilization when administered orally. The rats used for this study were fed the fat-free diet for four weeks. They were clearly EFA deficient, as judged from the low 18:2ω6 and 20:4ω6 and high 20:3ω9 acid contents of their liver PL, compared to the rats on the EFA-adequate diet.

A three-day administration of EFA was chosen to test whether short-term rectal administration of linoleic acid-rich MG is feasible and effective, and because changes in the fatty acid composition of liver lipids of EFA-deficient rats were already apparent within 24 hr after linoleic acid administration (23). Full recovery including restoration of 20:4ω6 to normal values would have taken much longer (24). The amount of linoleic acid administered represents about 0.32% of the calories, and the 7-wk-old EFA-deficient rats consumed about 11 g of fat-free diet per day. This dose is 32% of the minimum daily requirement, is high enough to induce significant increases in PUFA in liver lipids and is in the linear portion of the dose-response curve, (25) where its effect is most easily detected and measured. Differences due to modes of administration should be maximally measurable in this range.

Oral administration of linoleic acid as corn oil induced changes in the fatty acid composition of liver and serum lipids that are characteristic of early recovery of EFA deficiency (26). Rectal administration of linoleic acid-rich MG induced several significant changes in fatty acid parameters, which in most instances were very similar in magnitude to the effects induced by the same amount of linoleic acid given orally as corn oil or as corn oil-derived MG. This indicates that linoleic acid was efficiently absorbed when administered rectally as linoleic acid-rich MG.

Some of the linoleic acid administered rectally as MG may have been taken in orally due to the rat's habits of coprophagy and rectal preening. Coprophagy was minimized by housing rats individually in cages with wire bottoms (½″ mesh). Defecation was induced by the rat's being handled prior to dosing, and the small dose (27 mg) of viscous MG was introduced 1.5 cm into the empty rectum, minimizing the possibility of leakage to the exterior. The significant differences in the amounts of PL and FFA in liver after administration of MG either orally or rectally indicates oral uptake of rectally administered MG must be small, if there is any.

Rectal administration of linoleic acid-rich MG was more efficient in reducing the amount of PL fatty acids and in increasing the amount of FFA in liver than was oral administration of linoleate (Table 3). This finding demonstrates that the route of administration of linoleate may have an effect on lipid metabolism.

Almost all changes in fatty acid composition that assess early recovery from EFA deficiency (26) were found to be significantly and equally restored by rectal linoleic acid-rich MG and by oral administration of linoleic acid either as TG or MG. These results indicate that the rectal route may have potential for administration of EFA. Differences in physiology of absorption from the lower gastrointestinal tract are known to exist between rats and man. Studies in primates may be advisable before attempting rectal administration of EFA as MG to supplement stressed humans unable to take food by mouth.

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Platelet-Activating Factor Modulates Phospholipid Acylation in Human Neutrophils

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The present study showed that platelet-activating factor (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF), but not lysoPAF (1-O-hexadecyl-*sn*-glycero-3-phosphocholine) rapidly (within 15 sec) stimulated the incorporation of both [1-^{14}C]arachidonate and [1-^{14}C]docosahexaenoate into phosphatidylinositol (PI) and phosphatidylcholine (PC) in human neutrophils. Concomitantly, it inhibited the formation of labeled phosphatidic acid from both fatty acids. The magnitude of stimulation (percentage of control) was greater in PI than in PC for the incorporation of arachidonate and vice versa for the incorporation of docosahexaenoate. It reached a maximum at 10^{-7} M and started to decline at 10^{-6} M. Extracellular Ca^{2+} was not essential for the action of PAF on phospholipid acylation. The distribution of labeled arachidonate in the molecular species of PC was not altered by PAF after 1 min incubation, suggesting that the increased formation of arachidonyl-PC during the early stage of neutrophil-PAF interaction was not originated from the added PAF. No measurable changes in the mass of each phospholipid were detected in neutrophils challenged by PAF from 15 sec to 2 min. The data suggest that the increased incorporation of extracellular fatty acids into PI and PC elicited by PAF may be secondary to increased deacylation of these phospholipids, and the magnitude of stimulation reflects the specificity of acyltransferase catalyzing the acylation of lysoPI and lysoPC by fatty acyl-CoA.
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Platelet-activating factor (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF) is a bioactive phospholipid with multiple biological effects (1–4). At nanomolar concentrations PAF stimulates human neutrophil chemotactic migration, superoxide production, aggregation, cytochalasin B-dependent exocytosis of azurophilic and specific lysosomal granules (5,6). It enhances human neutrophil superoxide generation in response to chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) (7). This bioactive phospholipid also modulates the metabolism of other phospholipids in neutrophils. It enhances the activity of polyphosphoinositide-specific phospholipase C in rabbit peritoneal neutrophils (8), the release of arachidonate from phosphatidylinositol (PI) and phosphatidylcholine (PC) by the action of phospholipase A_2 and the production of 5-hydroxyeicosatetraenoate (5-HETE) and 5,12-dihydroxyeicosatetraenoate or leukotriene B_4 (LTB_4) in cytochalasin B-treated rabbit peritoneal neutrophils (9). It also promotes arachidonate incorporation into PI and PC in neutrophils from guinea pig peritoneal exudates (10). Since neutrophils from human blood differ from elicited neutrophils in a number of respects (11–13), it is important to examine whether the increased incorporation of arachidonate into PI and PC occurs in human neutrophils in response to PAF.

Recently docosahexaenoate was found to be incorporated into PC and phosphatidylethanolamine (PE) by human neutrophils (14). This n-3 fatty acid was a relatively poor substrate for incorporation into PI compared to arachidonate and eicosapentaenoate (14). It is thus important to examine whether the incorporation of docosahexaenoate into phospholipids in these cells is responsive to PAF. The present study has demonstrated that PAF rapidly increases (within 15 sec) the incorporation of both arachidonate and docosahexaenoate into PI and PC by human neutrophils.

MATERIALS AND METHODS

Preparation of human neutrophils. Human blood was obtained from healthy donors who had received no medication in the previous two weeks. Neutrophils were isolated according to the method of Lee et al. (15). Thirty-ml portions of venous blood were each mixed with 4 ml of 0.15 M sodium citrate, pH 5.2, and 5 ml of 5% dextran T500 (Pharmacia Fine Chemicals, Piscataway, New Jersey) in 0.15 M NaCl and were allowed to sediment at room temperature for 30 min. The supernatants containing leukocyte-rich plasma were removed and centrifuged at 250 × g for 10 min at 20 C. After hypotonic lysis of contaminating erythrocytes, leukocytes were washed once and resuspended in a modified Krebs-Ringer HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) buffer (118 mM NaCl, 4.74 mM KCl, 1.19 mM $MgSO_4$, 5 mM glucose, 16.3 mM HEPES, pH 7.4) at 20 × 10^6 cells/ml. Six-ml cell suspensions were layered on 6-ml Ficoll-Hypaque (Pharmacia) cushions and centrifuged at 400 × g for 20 min at 20 C to yield a neutrophil pellet, which was washed twice and suspended at a concentration of 20 × 10^6 cells/ml in the same buffer. Cell counts were made in a hemocytometer, and cell viability was measured by trypan blue exclusion and by lactate dehydrogenase release into the incubation medium as described previously (16). Cell preparations contained more than 95% neutrophils.

Incubation of cells. [1-^{14}C]Arachidonic acid (54.5 Ci/mol, New England Nuclear Corp., Boston, Massachusetts) or [1-^{14}C]docosahexaenoate (55.0 Ci/mol, New England Nuclear) was dissolved in 0.9% NaCl containing fatty acid–free bovine serum albumin (Miles Laboratories, Elkhart, Indiana; 4 mg/ml). PAF (1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, Novabiochem, Läufelfingen, Switzerland), lysoPAF (1-O-hexadecyl-*sn*-glycero-3-phosphocholine, Novabiochem) or lysoPC (1-acyl-*sn*-glycero-3-phosphocholine, Applied Science Laboratories, State College, Pennsylvania) was dissolved in 0.9% NaCl containing fatty acid–free bovine serum albumin (2.5 mg/ml). In a final volume of 2 ml, each tube contained 3.4 × 10^5 dpm (1.4 μM) [1-^{14}C]arachidonic acid or 3.6 × 10^5 dpm (1.5 μM) [1-^{14}C]docosahexaenoic acid, 1 mM $CaCl_2$, 0–10^{-6} M PAF (or 10^{-7} M lysoPAF or lysoPC) and 20 × 10^6 cells in 1.92 ml Krebs-Ringer HEPES buffer. In control tubes, an equivalent volume of 0.9% NaCl containing bovine serum albumin (2.5 mg/ml) was included. Incubations were initiated by the addition of 20 × 10^6 cells to each tube and were performed at 37 C.

Lipid extraction and analysis. Incubations were terminated by the addition of 5 ml methanol to each tube. Lipids were extracted according to the method of Bligh and Dyer (17) and dissolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Individual phospholipids were resolved and analyzed as described previously (13). Since the phosphorus content of phosphatidic acid (PA) cannot be accurately measured, the radioactivity of individual phospholipids is expressed as dpm/6 × 10^6 cells.

The distribution of [^{14}C]arachidonate in the molecular species of PC was analyzed as described previously (14).

RESULTS

Figure 1 shows the time course of the effect of PAF on the incorporation of [1-^{14}C]arachidonate into phospholipids in human neutrophils. PAF stimulated the incorporation of [1-^{14}C]arachidonate into PI and PC at all time intervals (15 sec to 2 min). Concomitantly, it inhibited the formation of [1-^{14}C]arachidonoyl-PA. The magnitude of stimulation (percentage of control) varied widely with cells from individual donors, but an increased acylation of labeled arachidonate into PI and PC was consistently demonstrated after 15 sec incubation of cells with PAF. In two separate experiments, PAF at 10^{-7} M caused an increase in the radioactivity of PI and PC to 1278% and 287% of control, respectively, at 15 sec; and the radioactivity of PI and PC became 1888% and 1283% of control, respectively, at 1 min. The magnitude of stimulation on the formation of labeled PI and PC induced by PAF started to decline at 2 min incubation. The incorporation of arachidonate into PE was less responsive to the presence of PAF. An increased radioactivity of PE was observed only after 2 min incubation of neutrophils with PAF. Analysis of phospholipid classes by phosphorus determination showed no measurable changes in phospholipid composition during the neutrophil-PAF interaction. At all time intervals (15 sec to 2 min), the lipid phosphorus (μg) from 6 × 10^6 cells in the presence or absence of PAF (10^{-7} M) was 0.803 for PC; 0.580 for PE; 0.197 for phosphatidylserine (PS); 0.125 for PI; and 0.295 for sphingomyelin.

Figure 2 demonstrates the time course of the effect of PAF on the incorporation of [1-^{14}C]docosahexaenoate into phospholipids. PAF inhibited the incorporation of [1-^{14}C]-docosahexaenoate into PA, but it enhanced the incorporation of this n-3 fatty acid into PI and PC. In two separate experiments, the average radioactivities of PI and PC were increased to 403% and 880% of control, respectively, after 15 sec incubation and to 432% and 2118% of control, respectively, after 1 min incubation. The magnitude of stimulation by PAF on the formation of labeled PC but not PI started to decline after 2 min incubation.

PAF in a range of 10^{-10}–10^{-6} M elicited significant changes in the incorporation of [1-^{14}C]arachidonate into phospholipids after 1 min incubation (Fig. 3). Maximal incorporation of [1-^{14}C]arachidonate into PI and PC was observed at 10^{-7} M, and a decline in the stimulation of the acylation of both phospholipids occurred at 10^{-6} M. At the concentrations tested (10^{-9}–10^{-6} M), PAF decreased the formation of [^{14}C]arachidonoyl-PA.

PAF has been shown to promote Ca^{2+} uptake from extracellular medium by rabbit platelets (18) and rabbit peritoneal neutrophils (8). A requirement for extracellular Ca^{2+} has been demonstrated for the stimulatory effect of PAF on degranulation (8), but not on polyphosphoinositides-specific phospholipase C (8) or superoxide generation (19). To test if extracellular Ca^{2+} is required for the action of PAF on phospholipid acylation by polyunsaturated fatty acids, the incorporation of [1-^{14}C]arachidonate into phospholipids in cells incubated in the presence of 1 mM Ca^{2+} was compared with that in cells incubated in the presence of 1 mM EGTA [ethyleneglycol-bis-(β-aminoethylether)N,N′-tetracetic acid]. Table 1 shows

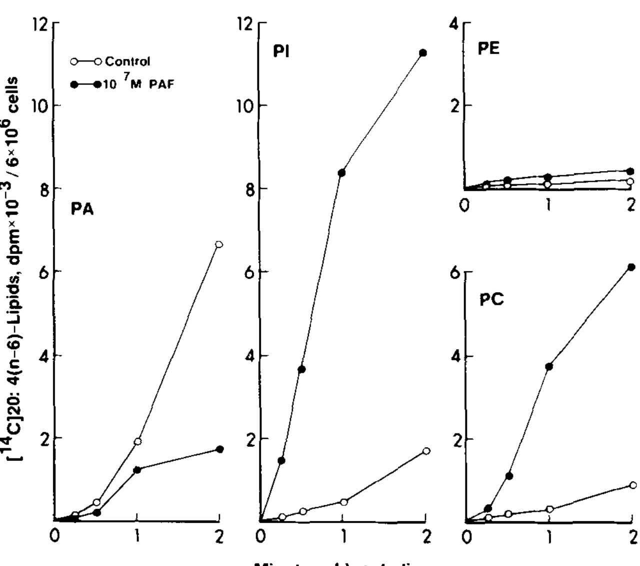

FIG. 1. Time course of the effect of PAF on the acylation of [1-^{14}C]arachidonate [20:4(n-6)] into phospholipids in human neutrophils. Neutrophils (20 × 10^6) were incubated at the indicated period of time (15 sec–2 min) with 3.4 × 10^5 dpm [1-^{14}C]arachidonate in the presence (●) or absence (○) of 10^{-7} M PAF. Each point represents the average value of duplicate incubations from two separate neutrophil preparations.

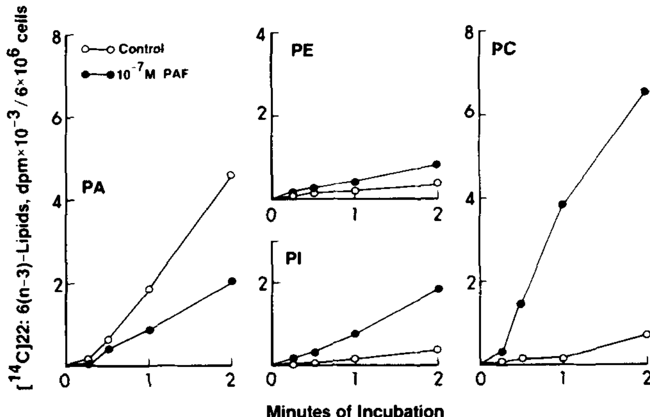

FIG. 2. Time course of the effect of PAF on the acylation of [1-^{14}C]docosahexaenote [22:6(n-3)] into phospholipids in human neutrophils. Neutrophils (20 × 10^6) were incubated at the indicated period of time (15 sec–2 min) with 3.6 × 10^5 dpm [1-^{14}C]docosahexaenote in the presence (●) or absence (○) of 10^{-7} M PAF. Each point represents the average value of duplicate incubations from two separate neutrophil preparations.

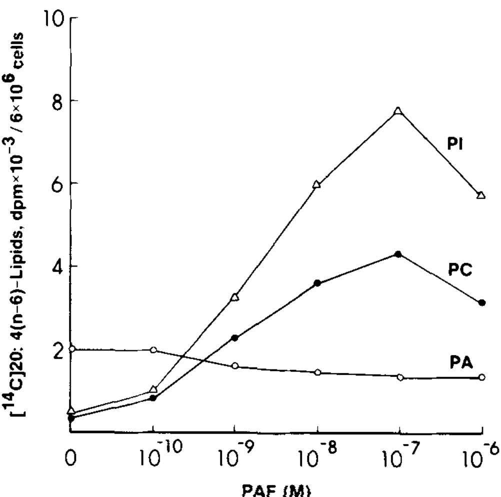

FIG. 3. Dose response to PAF for acylation of [1-^{14}C]arachidonate [20:4(n-6)] into phospholipids in human neutrophils. Neutrophils (20 × 10^6) were incubated for 1 min at 37 C with 3.4 × 10^5 dpm [1-^{14}C]-arachidonate and 0–10^{-6} M PAF. Each point represents the average value of duplicate incubations from two separate neutrophil preparations.

TABLE 1

Effect of External Ca^{2+} on [1-^{14}C]Arachidonate Incorporation into Phospholipids in Human Neutrophils in Response to PAF[a]

	Lipid	Radioactivity[b] (^{14}C dpm) Control	PAF	Mean % of control in the presence of PAF[c]
With Ca^{2+}	PA	2357	1146	57 ± 11
	PI	496	4558	1188 ± 377
	PC	383	2038	574 ± 122
Without Ca^{2+}	PA	1626	1184	66 ± 10
	PI	274	2676	1207 ± 350
	PC	296	1283	533 ± 152

PAF, platelet-activating factor; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine.

[a]Human neutrophils (20 × 10^6 cells) were incubated for 1 min at 37 C with [1-^{14}C]arachidonic acid (3.4 × 10^5 dpm, 1.4 μM), Ca^{2+} (1.0 mM) or EGTA (1 mM), and PAF (10^{-7} M). In control incubations, PAF was replaced by an equivalent volume of bovine serum albumin (2.5 mg/ml in 0.9% NaCl).

[b]The results from a typical experiment. The radioactivity is expressed as dpm from 6 × 10^6 cells.

[c]Mean values ± S.D. from three experiments.

that extracellular Ca^{2+} was not essential for the increased incorporation of [1-^{14}C]arachidonate into PI or PC induced by 10^{-7} M PAF after 1 min incubation. Although the basal radioactivity of phospholipids was less in the presence of 1 mM EGTA than that in the presence of 1 mM Ca^{2+}, PAF evoked a similar magnitude of stimulation in both systems.

The possibility that the increased radioactivity of PC was originated from the added PAF was examined. [^{14}C]Arachidonoyl-PC was treated with phospholipase C,

TABLE 2

Effect of Platelet-Activating Factor (PAF) on the Distribution of [1-^{14}C]Arachidonate in Diacyl-, Alkylacyl- and Alkenylacyl-phosphatidylcholine[a]

	% of Total [1-^{14}]arachidonate in phosphatidylcholine Diacyl	Alkylacyl	Alkenylacyl
Control	84.7	14.1	1.20
PAF (10^{-7} M)	82.9	15.7	1.40

[a]Human neutrophils (20 × 10^6 cells) were incubated for 1 min at 37 C with [1-^{14}C]arachidonic acid (3.4 × 10^5 dpm, 1.4 μM), Ca^{2+} (1.0 mM) and PAF (10^{-7} M). In control incubations, PAF was replaced by an equivalent volume of bovine serum albumin (2.5 mg/ml in 0.9% NaCl). Each phosphatidylcholine preparation was purified from 40 × 10^6 neutrophils and was treated with phospholipase C as described in Materials and Methods. The amount of radioactivity in each class of resulting diglyceride acetates is the average value from two separate experiments and is expressed as a percentage of the total radioactivity recovered from the thin layer plate.

TABLE 3

Effect of LysoPAF and LysoPC on the Incorporation of [1-^{14}C]Arachidonate into PI and PC by Human Neutrophils[a]

	^{14}C dpm (% of control) PA	PI	PC
Control	100	100	100
PAF (10^{-7} M)	50 ± 12	1200 ± 391	650 ± 182
LysoPAF (10^{-7} M)	100 ± 2.7	100 ± 0.7	101 ± 2.2
LysoPC (10^{-7} M)	78 ± 6.5	99 ± 3.2	140 ± 14

[a]PAF, platelet-activating factor; PC, phosphatidylcholine; PI, phosphatidylinositol; PA, phosphatidic acid. Human neutrophils (20 × 10^6) suspended in Krebs-Ringer HEPES buffer were incubated for 1 min at 37 C with [1-^{14}C]arachidonic acid (3.4 × 10^5 dpm, 1.4 μM); Ca^{2+} (1.0 mM); and PAF (10^{-7} M) or lysoPAF (10^{-7} M) or lysoPC (10^{-7} M). In control incubations, PAF was replaced by an equivalent volume of bovine serum albumin (2.5 mg/ml in 0.9% NaCl). Data represent the average value ± S.D. from four separate neutrophil preparations.

and the resulting 1-radyl-2-acylglycerols were acetylated. The 1-radyl-2-acyl-3-acetylglycerols formed were then resolved into 1-alkenyl-2-acyl-3-acetylglycerol, 1-alkyl-2-acyl-3-acetylglycerol and 1,2-diacyl-3-acetylglycerol, and the radioactivity in each fraction was measured as described previously (14). After 1 min incubation, more than 80% of the total radioactivity appeared in the 1,2-diacyl-3-acetylglycerol fraction from both control cells and cells exposed to PAF (Table 2), suggesting that the increased [^{14}C]arachidonoyl-PC was not derived from the added PAF under the experimental conditions. Exogenous lysoPAF (10^{-7} M) had no effect on the formation of [^{14}C]arachidonoyl-PC after a 1-min incubation, whereas exogenous lysoPC (10^{-7} M) increased the formation of [^{14}C]arachidonoyl-PC and concomitantly decreased the formation of labeled PA (Table 3). On the other hand, the

presence of lysoPC in the incubation medium exhibited no influence on the incorporation of [1-^{14}C]arachidonate into PI.

DISCUSSION

Increased incorporation of arachidonate into PI has been shown to occur in neutrophils in response to fMet-Leu-Phe in the presence of cytochalasin B (20), calcium ionophore A23187 (20), phorbol myristate acetate (21) and phagocytosis of inert particles (21). However, the present study demonstrated a rapid onset (within 15 sec) of increased incorporation of both [1-^{14}C]arachidonate and [1-^{14}C]docosahexaenoate into PI and PC during human neutrophil-PAF interaction. It also demonstrated that PAF inhibited the formation of labeled PA from both fatty acids. These findings suggest that the increased formation of labeled PI and PC was not brought about by de novo synthesis but by increased acylation of lysoPI and lysoPC. They also suggest that in the presence of PAF, cellular arachidonate and docosahexaenoate were shunted to the acylation of lysoPI and lysoPC from the acylation of lysoPA. It remains to be determined whether the labeled PA participates in the formation of PI and PC in control neutrophils, as an arachidonate- or docosahexaenoate-labeled cytidine diphosphodiacylglycerol has not been identified, and previous studies have shown only a low level of [^{14}C]docosahexaenoyl-1,2-diacylglycerol in intact human neutrophils (14). The formation of docosahexaenoyl-PA does not seem to be unique in intact neutrophils; it has been demonstrated in bovine retinal microsomes (22).

The magnitude of stimulation by PAF on the formation of labeled PI and PC appears to be a reflection of the specificity of the acyltransferase catalyzing the acylation of lysoPI and lysoPC by fatty acyl-CoA. It was greater in PI than in PC for the incorporation of arachidonate and vice versa for the incorporation of docosahexaenoate. In human neutrophils, arachidonate was a better substrate for incorporation into PI than into PC (9,13,23, 24), whereas docosahexaenoate was a relatively poor substrate for incorporation into PI as compared to arachidonate (14).

A concentration-dependent stimulation by PAF of the incorporation of arachidonate into PI and PC demonstrated in the present study was also observed in guinea pig peritoneal neutrophils (10) and in lysosomal enzyme secretion by human neutrophils (5). The decreased responsiveness at PAF concentrations above 10^{-6} M does not appear to be caused by damage of cell membranes, as no increase in the release of lactate dehydrogenase was detected in the incubation medium. It could be caused by a rapid neutrophil desensitization for limiting the overall cellular response.

Extracellular Ca^{2+} was not found to be essential for the increased incorporation of [1-^{14}C]arachidonate into PI and PC induced by PAF in human neutrophils under the experimental conditions. It was required, however, for the stimulation by PAF of the formation of arachidonoyl-PC but not arachidonoyl-PI in neutrophils from guinea pig peritoneal exudates (10). The discrepancy in the sensitivity toward external Ca^{2+} for the increased formation of arachidonoyl-PC induced by PAF in cells from different species is not clear. It may reflect different Ca^{2+} sensitivity of acyltransferase and/or phospholipase A_2 from different cells, as the combined activities of these enzymes control the levels of lysoPC. Human neutrophils have a higher content of alkylacyl-PC than guinea pig peritoneal neutrophils (13,25), and a Ca^{2+}-independent phospholipase A_2 specific for alkylacyl-PC is present in human amniotic fluid (26). It remains to be studied whether a similar Ca^{2+}-independent phospholipase A_2 is present in human neutrophils besides the Ca^{2+}-dependent phospholipase A_2 (27).

PAF in human neutrophils (28), rabbit peritoneal neutrophils (29) and rabbit platelets (30,31) is metabolized to 1-O-alkyl-2-acyl-*sn*-glycero-3-phosphocholine after a deacetylation-reacylation cycle. Although added PAF caused no measurable changes in the distribution of labeled arachidonate in the diacyl-, alkylacyl- and alkenylacyl-PC after 1 min incubation, the acylation of lysoPAF derived from added PAF would contribute to the increased radioactivity of PC after a longer period of incubation. LysoPC but not lysoPAF was shown to be rapidly acylated by [1-^{14}C]arachidonate to form labeled PC. This finding is in accord with the study demonstrating that extracellular arachidonate was incorporated into diacyl-linked PC more rapidly than into the ether-linked class (13,24,32). An undetectable acylation of exogenous lysoPAF after 1 min incubation could also be due to a slower rate of lysoPAF uptake by neutrophils, as demonstrated in rabbit platelets (31). An acylation of exogenous lysoPC into cellular PC was also demonstrated in rabbit peritoneal neutrophils (33) and in human mixed leukocytes from patients with chronic myelogenous leukemia (34).

The increased incorporation of fatty acids into PI and PC during the neutrophil-PAF interaction may be secondary to increased deacylation of these phospholipids by the action of phospholipase A_2, because no measurable changes in the mass of each phospholipid were detected under the experimental conditions. It remains to be determined whether the activity of the acyltransferase(s) was increased during the neutrophil-PAF interaction in view of the rapid onset of increased formation of labeled PI and PC. An increased acylation of lysoPI and lysoPC by exogenous fatty acids in neutrophils in response to PAF may serve to replenish these phospholipids following deacylation. This may also divert exogenous arachidonate from the 5-lipoxygenase pathway, thereby attenuating the formation of LTB_4 and 5-HETE, both of which are mediators of inflammation (35,36).

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The Influence of Dietary Fat on the Lipogenic Activity and Fatty Acid Composition of Rat White Adipose Tissue

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The in vivo fatty acid synthesis rate, selected enzyme activities and fatty acid composition of rat white adipose tissue from animals fed semisynthetic diets of differing fat type and content were studied. All animals were starved for 48 hr and then refed a fat-free (FF) diet for 48 hr. They were then divided into three groups. One group was continued on the FF diet for 48 hr. Another group was fed a diet containing 44% of calories from corn oil (CO). The final group was fed a diet containing 44% of calories from completely hydrogenated soybean oil (HSO). The animals on the FF diet had a marked increase in adipose tissue fatty acid synthesis during the 96-hr feeding period (as measured by ^{3}H incorporation into adipose fatty acids). Addition of either CO or HSO to the diets did not significantly inhibit fatty acid synthesis in dorsal or epididymal adipose tissue. The activities of the enzymes' fatty acid synthetase, ATP-citrate lyase and glucose-6-phosphate dehydrogenase increased on the FF diet and generally were not inhibited significantly by the addition of either fat to the diets. Linoleic acid was the major polyunsaturated fatty acid (ca. 22%) in adipose tissue. Monounsaturated fatty acids (palmitoleic, oleic, *cis*-vaccenic) made up ca. 38% of the total adipose fatty acids, while saturated fatty acids accounted for about 32% (myristic, palmitic and stearic). White adipose tissue in mature male rats was a major depot for n-3 fatty acids. There were differences in the fatty acid composition of epididymal and dorsal adipose tissue, particularly in their content of long chain, polyunsaturated fatty acids with epididymal tissue containing more of these compounds than dorsal fat. The fatty acid composition of the white adipose tissue did not change significantly during fasting or 96 hr of refeeding the FF diets. The addition of HSO to the diet for 48 hr had little influence on the adipose tissue fatty acid composition, but the addition of CO to the diet caused a 7% increase in the dorsal adipose tissue linoleate content (as percentage of total dorsal adipose tissue fatty acids) within 48 hr compared to animals fed the stock diet and those starved for 48 hr. The fatty acid synthesis data indicated that adipose tissue in the rat can continue to be a source of de novo fatty acid synthesis in animals consuming high-fat diets.
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Adipose tissue is a major site of fatty acid synthesis in rats (1–4) as well as other mammalian species (5–8). However, the regulation of fatty acid synthesis in adipose tissue is extremely complex as it is influenced by many intrinsic and extrinsic factors, such as diet (3,4,5,9–12), age (13,14), hormones (15–17) and heredity (18). There is uncertainty about the role of dietary fat on the suppression of fatty acid synthesis in adipose tissue (19–21).

The fatty acid synthetic rates in white adipose tissue have been measured using various fatty acid precursors such as ^{14}C-glucose (22,23), ^{14}C-acetate (24) and tritiated water (25,26). This latter substance has become the label of choice to measure fatty acid synthesis because it avoids the confounding influence of the various metabolic pools and the recycling phenomenon of the carbon precursors (27,28). Nevertheless, when studying fatty acid synthesis in the whole animal, one encounters the problem that adipose tissue, while actively synthesizing fatty acids, is a repository for fatty acids synthesized de novo in the liver (29) as well as for fatty acids of exogeneous origin. Some investigators have attempted to resolve this matter by suppressing liver fatty acid synthesis by diet (24) or drugs (29), or by using short incubation periods (25).

In rats fed stock diets ad libitum, six major fatty acids (30)—palmitic, palmitoleic, stearic, oleic, *cis*-vaccenic and linoleic—constitute about 90% of the total fatty acids present in white adipose tissue. Linoleic acid is the major polyunsaturated fatty acid of rat white adipose tissue (31). Under appropriate conditions dietary fatty acids can influence the fatty acid composition of adipose tissue (32–34).

We have previously reported the effect of feeding fat-free (FF) and fat-containing diets on liver and plasma fatty acid composition (35), as well as the activity of liver lipogenic enzymes in rats fed these diets (36). The objective of this research was to investigate some of the parameters affecting the regulation of white adipose tissue fatty acid metabolism by dietary fat in mature rats fed a semisynthetic diet. Here we present data on the synthetic rate, some enzymes of the fatty acid synthetic pathway and the fatty acid composition of epididymal and dorsal adipose tissue as a function of time on the FF and high-fat diets. Under the conditions used in this study it was found that high-fat diets, both saturated and polyunsaturated, did not suppress fatty acid synthesis in adipose tissue.

MATERIALS AND METHODS

Materials, animals and diets. Male, Sprague-Dawley rats, 100 to 150 g, were purchased from Bantin and Kingman (Fremont, California). They were fed a stock diet from Ralston-Purina (Richmond, Indiana) until they had grown to 400 to 500 g in weight and were 120 to 140 days old. The animals were then segregated into three groups of 12 to 22 rats each, starved for 48 hr and then refed a FF diet (75% of calories from carbohydrates) for 48 hr. After that, one group was continued on the FF diet for another 48 hr. A second group was placed on a synthetic diet in which 44% of the calories was supplied by corn oil (CO). The remaining group was fed a diet in which 44% of the calories was supplied by completely hydrogenated soybean oil (HSO), a gift of Durkee Foods (Cleveland, Ohio) for 48 hr. The composition of the three diets has been reported previously (35). The fatty acid composition of the diet is listed in Table 1. Animals had access to the diets ad libitum, except that initially the animals were

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TABLE 1

Fatty Acid Composition of Experimental Diets (Wt % of Total Fatty Acids)

Fatty acid[a]	Corn oil	Hydrogenated soybean oil
14:0	0.12	0.12
16:0	11.23	10.72
16:1(n-7)	0.10	—
17:0	0.14	0.25
18:0	2.06	86.37
18:1(n-9)	25.55	0.32
18:1(n-7)	1.04	—
18:2(n-6)	55.02	—
18:3(n-3)	0.42	—
20:0	0.46	0.55
20:1(n-9)	0.25	—
20:4(n-6)	0.09	—
22:0	0.16	—
24:0	—	0.11
Sum of trace components	2.96	1.21

[a]Fatty acids are designated by carbon chain length, number of double bonds and position of the first double bond from the methylene end of the molecule.

given approximately 3 ml of each diet by stomach tube. All animals consumed approximately the same number of calories regardless of diet group and appeared to find the diets palatable.

The animals were killed at 0, 24, 48, 72 and 96 hr after refeeding was begun. All animals received intraperitoneally tritiated H_2O (0.5 mCi/100 g body weight) one hr before they were killed. The tissues were removed and handled as previously described (37).

All organic solvents were obtained from Burdick and Jackson (Muskegon, Michigan). Purified fatty acid methyl ester reference standards were purchased from Nu-Chek Prep (Elysian, Minnesota).

Lipid extraction. Hydroquinone was added to all samples during processing. Three adipose tissue samples from each dietary group were extracted using $CHCl_3$-MeOH (2:1, v/v) by the procedures described previously (37) after being lyophilized to remove all traces of free tritiated water. This procedure was used to determine the percentage of fat present in the tissue based on the wet weight of the tissue. The total lipid extracts (TLE) were either prepared for transmethylation immediately or stored under nitrogen at −20 C until further processing. The remaining samples, which were the bulk, were lyophilized and transmethylated directly as dried tissue samples as described below.

Transmethylation. The transmethylations of the TLE to obtain the fatty acid methyl esters (FAME) were carried out using methanolic HCl (7%, w/w) (37) as previously described. For the direct transmethylation of the freeze-dried adipose tissue sample, the following procedure was used: Ca. 25 to 50 mg of dry tissue was placed directly in a 30-ml screw-capped culture tube to which a reflux condenser and drying tube were attached. Methanolic HCl (5 ml) was then added to the vial, and it was heated under reflux at 85 C for 2 hr. It was cooled to room temperature and processed similarly to the transmethylation of an aliquot of the TLE (37).

After transmethylation the FAME were extracted into hexane, purified and diluted in hexane to an appropriate concentration as described previously (37). They were then stored under N_2 at −20 C until analyzed by gas liquid chromatography (GLC) or by liquid scintillation count of the ^3H in the fatty acid moiety.

GLC of FAME. The FAME samples were analyzed as described previously (37) on fused-silica capillary columns coated with SP-2340 (Supelco, Bellefonte, Pennsylvania). Samples were chromatographed on a Perkin-Elmer Sigma 2000 coupled to a P-E 7500 computer loaded with a Chrom 3 data analysis program.

The quantitative accuracy of the GLC procedures was evaluated by using either purified single FAME or reference mixtures selected to cover the range of FAME present in the experimental samples. Accuracy of the analysis was estimated to be within 5% for the major components (greater than 10% of the total FAME in the sample) and within 10% for the minor components in the samples.

The cholesterol extracted into the hexane washes of the transmethylation procedure was not separated from FAME prior to injection of the samples in the chromatograph. However, cholesterol is largely decomposed during acidic transmethylation (38), and free cholesterol does not elute as a discrete peak from an SP-2340 column under the conditions used in this work (37).

The compositions (wt %) of the FAME in the rat samples were derived from the area percentages of the chromatograms as described previously (37). A chromatogram had between 30 and 50 discrete FAME peaks. Most minor peaks individually contributed less than 0.1% to the total area and were not identified with confidence. The major identified components usually comprised 97% to 99% of the total FAME present in the sample. For convenience, only selected major fatty acids are listed in the tables. The minor fatty acids are collectively presented as "sum of trace components," which varies from 4–6% of the total fatty acids present in the samples. Both identified and unidentified components are grouped together in this category.

Enzyme analysis. Adipose tissue samples were homogenized at 0 C in 4 volumes of HEPES (0.2 M)/EDTA (0.1 mM)/2-mercaptoethanol (10 mM) buffer, pH 7.4. The homogenates were centrifuged at 20,000 × g for 1 hr at 4 C. Supernatants recovered after this centrifugation were used for enzyme and protein determinations. Activities of fatty acid synthetase (FAS) were determined as previously reported (39). A unit of FAS is taken as the activity of enzyme required to synthesize 1 nmol of palmitic acid (equivalent to the oxidation of 14 nmol of NADPH)/min at 30 C. The activity of ATP-acid citrate lyase (ACL) was determined by the method of Linn et al. (40). One unit is defined as the activity necessary to catalyze the oxidation of 1 μmol NADH/min. Glucose 6-phosphate dehydrogenase (G6PDH) activity was determined by the procedures described previously (39). One unit of G6PDH represents the activity of enzyme necessary for the production of 1 μmol NADPH/min at 30 C. Activities of all enzymes are expressed as mU/mg protein.

Protein contents of tissue homogenates were determined by the method of Lowry et al. (41). All statistical

analysis of the data were done using the two-tailed student's t-test.

RESULTS

Starving the animals for 48 hr suppressed fatty acid synthesis and lipogenic enzyme activities in white adipose tissue compared to the animals fed the stock diet ad libitum, but had no influence on the fatty acid composition of the adipose tissue. Refeeding a FF diet stimulated adipose tissue lipogenic activity markedly by 48 hr to levels above that found in rats fed the stock diet and again without influencing the fatty acid composition of this tissue. Figure 1 presents the time course of fatty acid synthesis in rat dorsal adipose tissue. The animals on the FF diet exhibited an almost linear increase in fatty acid synthesis during the course of the study. No significant change in the rate of synthesis in the dorsal fat was detected when either corn oil or hydrogenated soybean oil was added to the diet at 48 hr. However, the variance in the experimental values was much larger in the groups receiving the high-fat diets. Nevertheless, there were no significant differences between the means of the fatty acid synthesis rates among any of the three diet groups at 72 or 96 hr.

Figures 2, 3 and 4 show the level of activity of the three enzymes measured in dorsal adipose tissue for the same time points as in Figure 1. Again, while individual variations were large, generally no significant differences were detected between the three dietary groups at 72 and 96 hr with the exception that animals on the HSO diet had suppressed FAS activity at 96 hr compared to animals on the FF and CO diets. All three enzymes showed increased activity after refeeding a FF diet compared to animals on the stock diet or those starved for 48 hr.

Epididymal adipose tissue enzymes were also investigated and showed approximately the same pattern as found in the dorsal tissue except that the activity levels were somewhat lower than those observed in the dorsal fat. The incorporation of ^{3}H into the fatty acids of epididymal fat was also about 50% lower than that observed

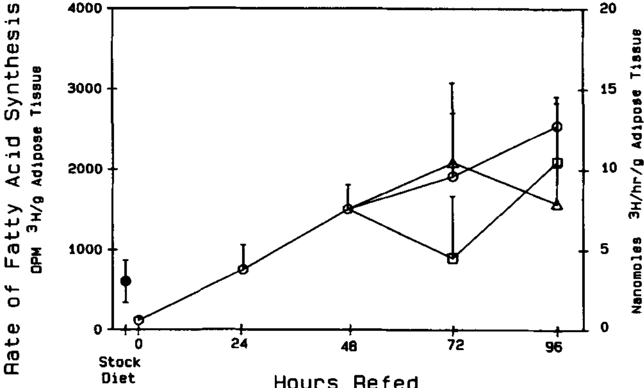

FIG. 1. Time course of fatty acid synthesis rate in dorsal adipose tissue from rats fed the three experimental diets. Symbols used are ●, stock diet; ○, fat-free diet; □, corn oil diet; △, hydrogenated soybean oil. Points are the means ± S.E.M. for groups of four to six animals. No statistically significant differences were detected between synthetic activities in animals on the fat-free diet and those on either the corn oil or hydrogenated soybean oil diets.

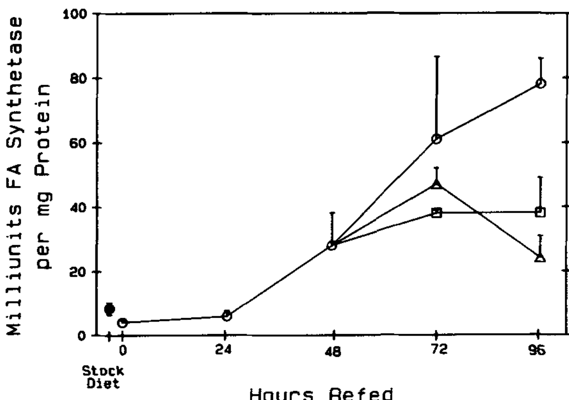

FIG. 2. Effect of different diets on the fatty acid synthetase activity in rat dorsal adipose tissue as a function of time on the diets. Symbols are the same as those in Fig. 1. The only difference that was statistically significant from the fat-free diet group was the 96-hr point for the hydrogenated soybean oil fed group, where $p < 0.01$.

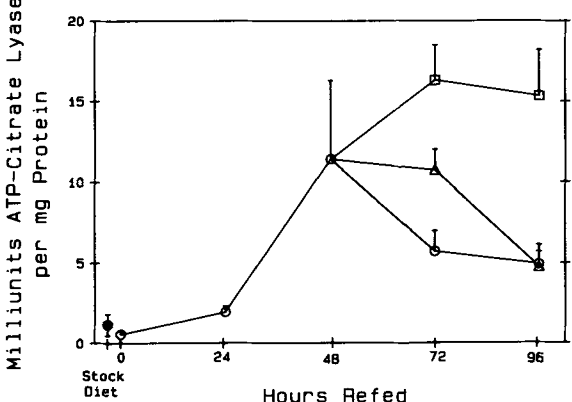

FIG. 3. Effect of different diets on ATP-citrate lyase activity in rat dorsal adipose tissue as a function of time on the diets. Symbols are as given in Fig. 1. Trends did not reach statistical significance during the 96-hr feeding period.

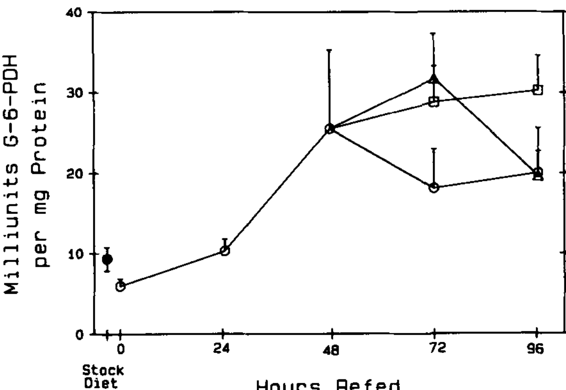

FIG. 4. Effect of different diets on the glucose-6-phosphate dehydrogenase activities in rat dorsal adipose tissue as a function of time on the diets. Symbols are the same as given in Fig. 1. No significant differences in the levels of enzyme activity were found among the tissues from animals in the three dietary groups.

in dorsal fat at equivalent time points. Actual data for the epididymal fat are not shown.

Table 2 gives the fatty acid composition of rat dorsal fat as a function of time and type of diet. There is relatively little change in the fatty acid composition between the animals starved for 48 hr and those refed a FF diet for 96 hr. There is a trend toward a reduction in the proportion of linoleate in adipose tissue after 96 hr on the FF diet, but this trend did not reach statistical significance. The only statistically significant change in any of the fatty acids in dorsal fat between the control animals and those on the FF diet was the 0.7% increase in *cis*-vaccenic acid at 96 hr.

When corn oil was added to the animals' diet after 48 hr on the FF diet, there was a statistically significant increase in the proportion of linoleate in dorsal fat. After 96 hr (48 hr on the CO diet) the linoleate level was 9% above that observed in the dorsal fat of the animals on the FF diet and 7% above the level in the dorsal fat of the animals starved for 48 hr. Other fatty acids showed statistically significant changes at 48 hr on the CO diet as well (see Table 2). Conversely, when saturated fat was added to the diet, there was little or no change in the fatty acid composition of the dorsal fat. Slight increases in the mean values for stearate, oleate and *cis*-vaccenic acids were noted in the dorsal fat of the animals fed saturated fat compared to animals on the stock diet or those starved for 48 hr. However, except for *cis*-vaccenic acid, the values were not significantly different at 96 hr.

It can also be observed from Table 2 that adipose tissue in the rat contains less than 1% arachidonic acid. Indeed, there is generally less arachidonic acid in adipose tissue than 22:6(n-3).

Table 3 lists the fatty acid composition of epididymal fat as a function of time and type of diet. Epididymal adipose tissue contains significantly larger proportion of polyunsaturated fatty acids than dorsal fat. In animals fed stock diets, the ratio of archidonic acid to the sum of n-3 20 and 22-carbon fatty acids is 0.34, while in epididymal fat this ratio is 0.25. Thus, there is almost 30% more n-3 fatty acids in epididymal fat proportionally to arachidonic acid than in dorsal fat.

DISCUSSION

It is generally agreed that dietary linoleic acid suppresses liver fatty acid synthesis (1,4,9,42–45), but several previous reports suggest that saturated fat does not suppress liver fatty acid synthesis (9,10,21,43,45–47). We recently reported (36) that in starved refed rats, high saturated-fat diets suppressed liver fatty acid synthesis equivalent to linoleate-containing diets. Some investigators, using various feeding periods (9,48), have suggested that fatty acid synthesis in adipose tissue is not inhibited by dietary fat. Reports on the enzymes of fatty acid synthesis indicate that rat adipose tissue fatty acid synthetic enzymes (usually measured in the epididymal fat pad) are not suppressed by dietary fat (5,10,17,21,49), although these studies used feeding periods and animals of ages different from those in this work. No consistent suppression of fatty acid synthesis was noted here in either dorsal or epididymal adipose tissue, even though linoleic acid was found to be rapidly incorporated into adipose fat. This suggests that other modulators of fatty acid synthesis, such as blood glucose levels, may play an important role in triggering fatty acid synthesis in adipose tissue.

Recently Gandemer et al. (25), using tritiated water and short incubation times to minimize the contribution from fatty acids synthesized de novo by the liver, indicated that in rats on a low-fat diet (0.3%) only 27% of the total fatty acids was synthesized in total body adipose tissue, whereas 42% of the fatty acid synthesis took place in the liver. Earlier work by Clarke and coworkers (4,9) and Romsos and Leveille (19) in meal-fed rats suggested that as much as 70% of the de novo fatty acid synthesis in the rat took place in the adipose tissue. High levels of dietary fat almost totally suppress liver fatty acid synthesis (9,21,36,46,50), and there is little if any transport of de novo synthesized fatty acids in the circulation by this time (35). Thus, the contribution of fatty acids synthesized in the liver to adipose tissue fatty acid stores should be minimal in rats on a high-fat diet.

It may be that a fat-fed animal synthesizes fatty acids primarily in the adipose tissue while those on a very low or fat-free diet synthesize fatty acids primarily in the liver with, perhaps, a substantial synthetic contribution from the adipose tissue. Factors including diet, age, sex, strain and caloric intake probably all affect the site of fatty acid synthesis, so considerable caution must be exercised in assigning the relative contribution of the liver or adipose tissue to de novo fatty acid synthesis in the rat. Meal feeding vs ad libitum feeding will also affect this ratio (48).

The fatty acid compositional data shown in Tables 2 and 3 were obtained by direct transmethylation of the lyophilized tissue. No compositional differences in the adipose fatty acid data could be detected between portions of the same adipose tissue sample processed by direct transesterification of the lyophilized tissue or by transesterification of the TLE from that tissue. The direct method was simple and quantitative, and saved considerable time and effort by avoiding the initial extraction step using $CHCl_3$/MeOH. This method works well for adipose tissue probably because adipose tissue is about 90% triglyceride and, hence, there are few proteins, carbohydrates or other nonlipoidal substances present to interfere with the reaction. Other investigators have also recently proposed one-step transesterification procedures (51,52) applicable to a variety of tissues. However, no attempt was made in this work to determine if this method was applicable to other tissues.

Palmitic, palmitoleic, stearic, oleic, *cis*-vaccenic and linoleic acids constitute almost 90% of total fatty acids (30,32,53) in adipose tissue. This may be true regardless of diet (30,31,54), which suggests that there are regulatory mechanisms controlling the fatty acid composition of this tissue other than the fatty acid composition of the diet. By comparing Tables 2 and 3 one can find subtle differences between the fatty acid compositions of the dorsal and epididymal fat. They may be due to differences in the amounts of phospholipids present in the two tissues, but any physiological significance of these differences remains obscure.

While dietary linoleic acid causes a rapid increase in the linoleic acid content of white adipose tissue, a high level of stearic acid in the diet has little influence on the content of this fatty acid in adipose tissue. The stearic acid level in adipose tissue is low, presumably due to the active Δ-9 dehydrogenase present in adipose tissue (33,54).

TABLE 2

Fatty Acid Composition of Rat Dorsal Adipose Tissue (Wt % of Total Fatty Acids)

Fatty acid[a]	Ad-libitum fed stock diet (n = 5)	48 Hr starved 0 Hr (n = 4)	Fat-free, high carbohydrate diet				Corn oil diet (after 48 hr on FF diet)		Saturated fat diet (after 48 hr on FF diet)	
			24 Hr (n = 5)	48 Hr (n = 5)	72 Hr (n = 4)	96 Hr (n = 4)	24 Hr (n = 4)	48 Hr (n = 4)	24 Hr (n = 6)	48 Hr (n = 6)
14:0	1.98 ± 0.13	1.96 ± 0.13	1.92 ± 0.11	2.03 ± 0.25	1.84 ± 0.08	1.97 ± 0.13	1.72 ± 0.10	1.45 ± 0.32[b]	1.94 ± 0.14	1.89 ± 0.10
16:0	25.92 ± 1.86	25.24 ± 0.78	24.71 ± 1.19	25.89 ± 1.86	25.25 ± 0.44	25.78 ± 1.08	24.31 ± 1.80	22.33 ± 2.48[b]	24.92 ± 1.21	25.33 ± 1.33
16:1(n-7)	5.13 ± 1.26	3.85 ± 0.43	4.42 ± 1.05	5.07 ± 0.91	4.78 ± 0.68	5.73 ± 0.42	4.91 ± 0.97	3.30 ± 1.11[b]	5.31 ± 0.90	5.09 ± 0.94
18:0	4.85 ± 1.26	5.10 ± 0.55	4.97 ± 0.49	5.06 ± 0.33	5.08 ± 0.24	4.57 ± 0.18	4.70 ± 0.31	4.74 ± 0.13	5.06 ± 0.37	5.06 ± 0.61
18:1(n-9)	29.63 ± 1.61	29.17 ± 1.08	28.72 ± 1.20	29.85 ± 0.93	29.50 ± 1.30	29.60 ± 0.49	31.57 ± 1.32	29.89 ± 0.88	30.71 ± 1.31	30.31 ± 0.23
18:1(n-7)	3.58 ± 0.22	3.89 ± 0.12	4.07 ± 0.21	4.03 ± 0.29	4.05 ± 0.19	4.34 ± 0.24[b]	3.90 ± 0.15	3.42 ± 0.50	4.33 ± 0.53	4.30 ± 0.47
18:2(n-6)	21.19 ± 3.96	22.71 ± 0.46	22.49 ± 2.16	19.98 ± 2.92	21.35 ± 0.99	19.76 ± 1.06	22.24 ± 2.88	28.76 ± 4.87[b]	20.49 ± 2.01	20.60 ± 1.70
18:3(n-3)	1.36 ± 0.34	1.29 ± 0.14	1.24 ± 0.14	1.13 ± 0.18	1.22 ± 0.15	1.16 ± 0.05	1.00 ± 0.10	0.90 ± 0.24[b]	1.03 ± 0.17	1.07 ± 0.09
20:4(n-6) + 22:1(n-11)	0.43 ± 0.11	0.42 ± 0.09	0.44 ± 0.11	0.40 ± 0.07	0.47 ± 0.13	0.46 ± 0.07	0.37 ± 0.12	0.48 ± 0.10	0.42 ± 0.09	0.44 ± 0.07
20:5(n-3)	0.27 ± 0.06	0.16 ± 0.05	0.20 ± 0.10	0.18 ± 0.03	0.19 ± 0.08	0.20 ± 0.05	0.09 ± 0.05	0.10 ± 0.05[b]	0.11 ± 0.05	0.11 ± 0.03
20:5(n-3)	0.37 ± 0.08	0.37 ± 0.10	0.41 ± 0.17	0.33 ± 0.09	0.42 ± 0.16	0.37 ± 0.07	0.23 ± 0.09	0.19 ± 0.12[b]	0.32 ± 0.16	0.31 ± 0.17
22:6(n-3)	0.62 ± 0.24	0.66 ± 0.15	0.79 ± 0.20	0.61 ± 0.16	0.74 ± 0.31	0.79 ± 0.19	0.38 ± 0.13	0.34 ± 0.21	0.55 ± 0.23	0.64 ± 0.17
Sum of trace components	4.67 ± 1.24	5.24 ± 0.96	5.62 ± 0.85	5.44 ± 1.06	5.11 ± 0.89	5.27 ± 1.29	4.58 ± 1.13	4.62 ± 0.96	4.81 ± 0.87	4.85 ± 0.92

[a]Fatty acids designated by chain length, number of double bonds and position of the first double bond from the methylene end of the molecule.
[b]Values significantly different from those for animals fed the stock diet or starved for 48 hr at $p < 0.05$.

TABLE 3

Fatty Acid Composition of Rat Epididymal Adipose Tissue (Wt % of Total Fatty Acids)

Fatty acid[a]	Ad-libitum fed stock diet (n = 5)	48 Hr starved 0 Hr (n = 4)	Fat-free, high carbohydrate diet		Corn oil diet (after 48 hr on FF diet	Saturated fat diet (after 48 hr on FF diet)
			48 Hr (n = 5)	96 Hr (n = 4)	48 Hr (n = 4)	48 Hr (n = 6)
14:0	1.74 ± 0.27	1.51 ± 0.14	1.82 ± 0.27	1.63 ± 0.12	1.38 ± 0.10[b]	1.62 ± 0.10
16:0	24.42 ± 1.92	23.04 ± 1.80	23.21 ± 1.33	23.64 ± 0.42	21.68 ± 0.78[b]	23.17 ± 0.87
16:1(n-7)	5.34 ± 1.82	4.53 ± 0.88	4.67 ± 1.77	4.95 ± 0.65	3.57 ± 0.61[b]	5.14 ± 0.96
18:0	4.73 ± 0.44	4.58 ± 0.51	5.01 ± 0.44	4.80 ± 0.39	5.01 ± 0.40	4.85 ± 0.55
18:1(n-9)	28.00 ± 0.57	27.96 ± 0.62	28.10 ± 1.39	28.88 ± 1.14	28.66 ± 0.64	28.96 ± 0.83
18:1(n-7)	3.88 ± 0.36	4.15 ± 0.16	4.08 ± 0.54	4.82 ± 0.25[b]	3.91 ± 0.31	4.58 ± 0.35
18:2(n-6)	21.45 ± 2.19	22.62 ± 1.20	22.58 ± 1.59	21.59 ± 1.14	26.50 ± 1.56[b]	22.50 ± 1.10
18:3(n-3)	1.41 ± 0.18	1.41 ± 0.10	1.45 ± 0.08	1.33 ± 0.08	1.16 ± 0.21[b]	1.32 ± 0.08
20:4(n-6) + 22:1(n-11)	0.77 ± 0.17	0.86 ± 0.11	0.74 ± 0.19	0.63 ± 0.09	0.75 ± 0.14	0.67 ± 0.10
20:5(n-3)	0.52 ± 0.12	0.51 ± 0.09	0.48 ± 0.21	0.36 ± 0.10	0.28 ± 0.10[b]	0.30 ± 0.08
22:5(n-3)	0.69 ± 0.18	0.87 ± 0.15	0.70 ± 0.24	0.54 ± 0.12	0.51 ± 0.13[b]	0.53 ± 0.11
22:6(n-3)	1.58 ± 0.41	1.94 ± 0.36	1.57 ± 0.56	1.26 ± 0.34	1.15 ± 0.34	1.25 ± 0.25
Sum of trace components	5.47 ± 1.31	6.02 ± 1.01	5.54 ± 1.21	5.57 ± 1.06	5.44 ± 1.02	5.06 ± 0.91

[a]Fatty acids designated by chain length, number of double bonds and the position of the first double bond from the methylene end of the molecule.
[b]Values significantly different from those for animals fed the stock diet or starved for 48 hr at $p < 0.05$.

It is possible that poor intestinal absorption of stearic acid is the reason that the hydrogenated fat diet had little influence on the fatty acid composition of the adipose tissue. However, all the animals in this study consumed approximately the same number of calories and gained about the same amount of weight. Additionally, 40% of the calories consumed in the hydrogenated fat diet were in the form of stearic acid, so that even a relatively poor absorption rate for this compound would still provide a significant amount of stearic acid for metabolic use as reported by Clarke et al. (9).

Most previous reports on the fatty acid composition of adipose tissue have not included data on the 20- and 22-carbon polyunsaturated fatty acids present in this tissue (30,34,55). In this work we observed significant amounts of 20:4(n-6), 22:5(n-3) and 22:6(n-3), and small, but measurable, amounts of 20:5(n-3) in both dorsal and epididymal fat. Indeed, the 22-carbon, n-3 polyunsaturated fatty acids constitute more of the adipose tissue fatty acids than arachidonic acid, an unusual circumstance in the rat, whose tissues usually contain relatively large amounts of arachidonic acid (35,56,57).

Adipose tissue is a major reservoir of fatty acids in the mature rat (22,57); indeed, the adipose tissue pool of fatty acids may be larger than all other carcass fatty acid pools combined (14,22,58). If all the individual long chain n-3 fatty acids in rat adipose tissue are summed, they represent between 3% and 4% of the total adipose fatty acids. This is in contrast to the arachidonic acid pool which, although much larger than the n-3 pool in rats, is concentrated in more physiologically active tissue, such as heart muscle (57,59), liver (35,37,60), nerve (61) and various others (53,57,61,62). The accumulation of n-3 fatty acids by adipose tissue may be an aging phenomenon as the 22-carbon, n-3 fatty acids are not precursors of physiologically active eicosanoids (63) and may accumulate in the adipose tissue with age.

There was no inhibition of the lipogenic enzymes, measured in white adipose tissue during the refeeding period of this study. When we fed corn oil to the animals, linoleic acid levels rose markedly in the adipose tissue within 48 hr, whereas feeding saturated fat had no influence on the fatty acid composition of the adipose tissue in the same time period. Feeding these two types of fat for longer periods could clarify whether these differences were due to slower absorption and/or transport of saturated fat or to different pathways for the metabolism of saturated versus polyunsaturated fatty acids in the rat.

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Effect of Age and Dietary Fat (Fish, Corn and Coconut Oils) on Tocopherol Status of C57BL/6Nia Mice

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The effect of age and dietary fat type on tocopherol status was investigated using young and old C57BL/6Nia mice fed semipurified diets containing 5% (by weight) fish, corn or coconut oils and supplemented with 30, 100 or 500 ppm dl-α-tocopheryl acetate for 6 wk. Tocopherol levels in the diets, plasma, liver, kidney and lung were measured by high performance liquid chromatography following appropriate extractions. The results indicate that mice fed fish oil maintain lower plasma and tissue tocopherol concentrations than those fed corn and coconut oils (fish < corn oil < coconut oil). The difference was not due to a loss of tocopherol prior to consumption, but rather appeared to occur during the absorption process. Old mice had lower plasma and liver tocopherol concentrations than young mice. Old mice fed fish oil, however, maintained plasma tocopherol levels better than young mice fed fish oil, presumably due to their larger tocopherol pool. No age effect was detected on kidney and lung tocopherol levels. It is concluded that tocopherol status is affected by age and dietary fat type, especially fish oil. *Lipids 22,* 345–350 (1987).

The dependency of tocopherol requirements on the level of polyunsaturated fats in the diet has been established (1–5). Most previous work, however, has concentrated on unsaturated oils of plant origin. Interaction of tocopherol with the polyunsaturated fatty acids of marine oils having higher degrees of unsaturation than those in plant oils has not been well studied. Data is especially lacking for aged animals, which possess more lipid peroxides (6,7) and might therefore be more susceptible to changes in antioxidant defense mechanisms such as tocopherol status.

The lower incidence of coronary heart disease in Greenland Eskimos (8) has been attributed to their high consumption of marine oils and their fatty acid components, especially eicosapentaenoic acid. Eicosapentaenoic acid is a precursor for the 3-series of prostaglandins and the 5-series of leukotrienes, which have been shown to have less potent aggregatory and inflammatory properties than the corresponding arachidonic acid metabolites (9, 10). These findings have renewed interest in fish oil and its potential use in the prevention and/or therapy of certain chronic cardiovascular and inflammatory diseases. However, over 50 years ago, Goettsch and Pappenheimer (11) reported extensive muscle lesions produced in rabbits fed diets supplemented with cod liver oil. Subsequently, Madsen et al. (12) showed that addition of cod liver oil to synthetic and natural food diets produced muscle lesions in rabbits, guinea pigs, goats and sheep. McKenzie et al. (13) later showed that this toxic effect of cod liver oil could be prevented by the administration of vitamin E.

The effect of fish oils on tocopherol status, which could influence some of the observed beneficial and toxic effects, has been mostly overlooked. In studies comparing the biological effects of fish oil with those of other dietary oils in mice, we noticed that serum tocopherol levels were different among different dietary groups, making interpretation of the data difficult. Therefore, the present study was conducted to compare the effect of fish oil on tocopherol status with that of corn and coconut oils in young and old mice.

MATERIALS AND METHODS

Animals and diets. Three-month (young) or 24-month (old) specific pathogen-free male C57BL/6Nia mice were purchased from the National Institute of Aging colonies at the Charles River Breeding Laboratories (Wilmington, Massachusetts) and were fed semipurified diets containing 5% (by weight) fat as either tocopherol-stripped corn oil, nonhydrogenated coconut oil (Tekland, Madison, Wisconsin) or fish oil (MAXEPA; a gift from R.P. Scherer Co., Troy, Michigan) and supplemented with 30, 100 or 500 ppm dl-α-tocopheryl acetate (Tekland) for 4 wk. The overall design of the study is shown in Figure 1; composition of the basal diet is shown in Table 1. Fresh diets were given daily just before the dark cycle (so that food did not sit in the jars for a long time before being consumed). Leftover food was discarded. The bulk of the diets, without oil, in powder form was prepared and stored at 4 C. Oils were mixed in smaller batches. Following addition of the oils, the diets were flushed with nitrogen and kept frozen. Samples of each diet were kept for tocopherol analysis.

Mice were housed individually in plastic cages with water and diets provided ad libitum. Weekly weights were obtained, and animals were checked daily for general health status.

Mice were anesthetized with nembutal (60 mg/kg BW) (Anthony Products Co., Arcadia, California). Blood was collected by heart puncture and plasma separated by cold centrifugation. Livers and kidneys were snap-frozen with

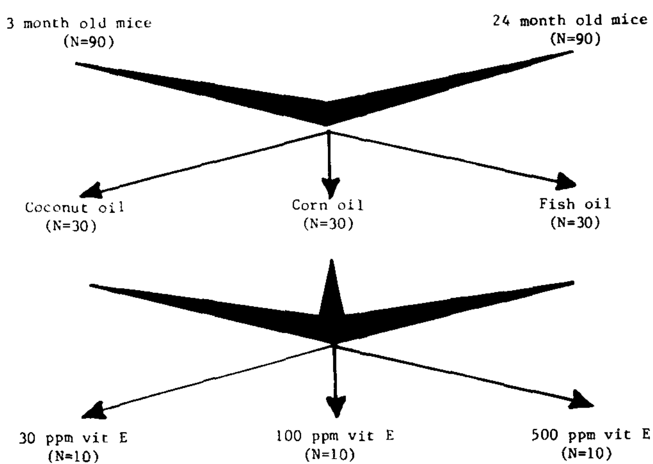

FIG. 1. Experimental design.

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TABLE 1

Composition of Basal Diet

Ingredient	% by Weight
Vitamin-free casein	18.00
Fat[a]	5.00
Corn starch	31.55
Sucrose	31.55
Cellulose	5.00
D-L methionine	0.30
Choline chloride	0.10
Salt mix (AIN-76)[b]	3.50
Vitamin mix[c]	1.00

[a]See text.

[b]The salt mix was supplemented with 0.0023 mg/kg diet of Na fluoride.

[c]Composition/kg vitamin mix: 150,000 IU vitamin A acetate; 15,000 IU vitamin D; 3,000 IU dl-α-tocopheryl acetate; 20 mg vitamin K (menadione); 20 mg biotin; 200 mg folacin; 2380 mg inositol; 3,000 mg niacin; 1,600 mg Ca pantothenate; 700 mg riboflavin; 600 mg thiamin; 700 mg vitamin B-6; 1 mg vitamin B-12; and up to 1 kg corn starch.

TABLE 2

Body Weights of 3-Month- and 24-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (g)	Corn oil (g)	Fish oil (g)
Young			
30	27 ± 1	25 ± 1	27 ± 1
100	26 ± 2	28 ± 1	29 ± 2
500	27 ± 1	27 ± 1	28 ± 1
Old			
30	32 ± 2	35 ± 2	33 ± 1
100	33 ± 2	34 ± 1	39 ± 1
500	32 ± 1	33 ± 1	35 ± 2

Mean ± SE, n = 10.

TABLE 3

dl-α-Tocopheryl Acetate Concentration in Corn Oil, Coconut Oil and Fish Oil Diets

Dietary fat	dl-α-Tocopheryl acetate added to diet (ppm)	dl-α-Tocopheryl acetate[a] measured in diet (ppm)		
		0 hr	6 hr	24 hr
Coconut oil	30	27 ± 3	36 ± 9	26 ± 4
Coconut oil	500	538 ± 17	(476)[b]	562 ± 18
Corn oil	30	34 ± 3	28 ± 8	26 ± 5
Corn oil	500	529 ± 5	521 ± 45	547 ± 69
Fish oil	30	69 ± 5	(58)[b]	70 ± 1
Fish oil	500	538 ± 17	524 ± 20	512 ± 39

Mean ± SE; n = 3 for 0 hr, n = 2 for 6 and 24 hr.

[a]Values have been calculated from α-tocopherol values obtained in extracted samples (after saponification of dl-α-tocopheryl acetate in the diet) multiplied by a correction factor calculated by dividing α-tocopherol MW/dl-α-tocopheryl acetate MW.

[b]Single measurement.

liquid nitrogen. All samples were kept at −70 C for α-tocopherol analysis.

α-Tocopherol analysis. α-Tocopherol content of diets, plasma and tissues was measured by the modified high performance liquid chromatography (HPLC) method of Bieri et al. (14). Plasma samples were deproteinized with ethanol containing 0.01% BHT. α-Tocopheryl acetate was used as an internal standard. After centrifugation, supernatant was extracted with 5 vol hexane. The hexane layer was removed, dried and redissolved in ethanol. Tissue samples (ca. 50 mg) were homogenized twice with 5 ml acetone. The acetone layers were pooled, evaporated under nitrogen and redissolved in ethanol. Tocopherol in food samples exposed to air for 0, 6 and 24 hr was analyzed following saponification in the presence of excessive amounts of ascorbic acid followed by hexane extraction as described by Taylor (15). α-Tocopherol in the extracts was separated using a 15-cm HPLC column packed with 5 μ C18 particles developed with 100% methanol. Elution spectra were detected at 292 nm excitation and 340 emission with spectrofluorometer.

Statistical analysis. Results were analyzed by three-way analysis of variance and were compared using statistical analysis system (SAS), least square mean comparison for the effects of age, fat type and vitamin E level on plasma and tissue α-tocopherol levels. They are recorded as mean ± standard error (SE).

RESULTS

Table 2 shows the body weights of young and old mice fed different diets. There was no significant effect of fat type or vitamin E level on body weight.

Table 3 shows the tocopherol concentration measured in different diets, fresh and following 6 or 24 hr exposure to air at room temperature, to account for losses that might occur prior to consumption. Each sample was a mixture of top, middle and bottom sections of the food container. Two or three samples were extracted from each container. There was no statistically significant loss of dl-α-tocopheryl acetate in the diet due to type of fat or time of exposure to room temperature. The higher tocopherol content in the fish oil diet containing 30 ppm vitamin E is due to tocopherol added by the manufacturer. Analysis of MAXEPA fish oil revealed 600 mg tocopherol/kg fish oil, which contributes ca. 30 ppm to the diet.

Tables 4 and 5 show the plasma tocopherol concentration in young and old mice fed different diets. Young mice fed fish oil had a lower plasma tocopherol level compared to mice fed corn and coconut oils at all three levels of tocopherol ($P < 0.0001$ for overall fat effect). Increasing the tocopherol level to 100 and 500 ppm in coconut oil and corn oil diets increased plasma tocopherol level over 100%, while only a 39% increase in plasma α-tocopherol was observed after increasing tocopherol levels to 500 ppm in the fish oil diet. A similar trend was observed in old mice (Table 5), except the differences were not as marked, especially in mice fed 30 ppm vitamin E. In

TABLE 4

Plasma Tocopherol Concentration in 3-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/dl)	Corn oil (μg/dl)	Fish oil (μg/dl)	
30	311 ± 25 (n = 10)	227 ± 27 (n = 9)	208 ± 17[b] (n = 8)	P < 0.07
100	476 ± 2[a] (n = 9)	450 ± 35[a] (n = 9)	237 ± 18[b] (n = 10)	P < 0.0001
500	756 ± 54[a] (n = 10)	592 ± 54[a,c] (n = 10	291 ± 42[b] (n = 7)	P < 0.0001
	P < 0.002	P < 0.0001	NS	

Mean ± SE. NS, not significant.
[a]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.
[b]Significantly different from mice fed coconut oil at P values indicated in the last column of each row.
[c]Significantly different from mice fed fish oil at P < 0.0001 and from mice fed coconut oil at P < 0.02.

TABLE 5

Plasma Tocopherol Concentration in 24-Month-Old C57BL/Nia Mice Fed Coconut Oil, Corn Oil or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/dl)	Corn oil (μg/dl)	Fish oil (μg/dl)	
30	294 ± 57 (n = 9)	254 ± 11 (n = 8)	285 ± 27 (n = 8)	NS
100	388 ± 48 (n = 8)	327 ± 26 (n = 7)	313 ± 21 (n = 7)	NS
500	472 ± 67[a] (n = 10)	470 ± 29[a] (n = 10)	329 ± 17[b] (n = 7)	P < 0.02
	P < 0.002	P < 0.0002	NS	

Mean ± SE. NS, not significant.
[a]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.
[b]Significantly different from mice fed coconut and corn oils at P value indicated in last column of each row.

TABLE 6

Liver Tocopherol Concentration in 3-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil, or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/g)	Corn oil (μg/g)	Fish oil (μg/g)	
30	38 ± 3 (n = 10)	11 ± 2[b] (n = 9)	7 ± 1[b] (n = 8)	P < 0.05
100	120 ± 15[a] (n = 10)	70 ± 12[a,b,c] (n = 9)	18 ± 2[b] (n = 10)	P < 0.009
500	310 ± 28[a] (n = 10)	109 ± 14[a,b,c] (n = 10)	23 ± 3[b] (n = 7)	P < 0.0001
	P < 0.0001	P < 0.002	NS	

Mean ± SE. NS, not significant.
[a]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.
[b]Significantly different from mice fed coconut oil at P values indicated in the last column of each row.
[c]Significantly different from mice fed fish oil.

TABLE 7

Liver Tocopherol Concentration in 24-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/g)	Corn oil (μg/g)	Fish oil (μg/g)	
30	26 ± 7 (n = 8)	11 ± 1 (n = 5)	13 ± 1 (n = 6)	NS
100	49 ± 8 (n = 7)	20 ± 3 (n = 7)	17 ± 3 (n = 7)	NS
500	99 ± 25[a] (n = 10)	69 ± 31[a] (n = 4)	28 ± 3[b] (n = 4)	P <0.004
	P < 0.0003	P < 0.05	NS	

Mean ± SE. NS, not significant.
[a]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.
[b]Significantly different from mice fed coconut oil at P values indicated in the last column of each row.

general, old mice fed corn and coconut oils had lower plasma tocopherol levels than young mice fed similar diets ($P < 0.05$ for overall age effect). This was not true for old mice fed fish oil. Plasma tocopherol levels in old mice fed fish oil, however, were lower than those in young mice fed corn or coconut oils. Correlation coefficients (r) between dietary and plasma tocopherol levels in young mice were 0.84, 0.73 and 0.39 and in old mice were 0.40, 0.80 and 0.27 for diets containing coconut oil, corn oil and fish oil, respectively.

Since the difference in plasma tocopherol levels in mice fed different dietary fats was not due to a loss of tocopherol in diet (see Table 3), tissue tocopherol concentration was examined as an indicator of differences in the absorption and/or utilization of tocopherol due to dietary fat type. Young and old mice fed fish or corn oils had lower liver tocopherol concentrations than those fed coconut oil ($P < 0.0001$ for overall fat effect). The fat effect was more pronounced in mice fed 100 and 500 ppm tocopherol (Tables 6 and 7).

In young mice fed coconut and corn oils, increasing the tocopherol level from 30 to 500 ppm in the diet increased liver tocopherol concentration almost 10-fold ($P < 0.0001$ and 0.0002, respectively), whereas mice fed fish oil showed only a threefold increase ($P > 0.8$). Livers from old mice, like plasma, had a lower tocopherol concentration than those of young mice, except for old mice fed fish oil ($P < 0.0001$ for overall age effect). Similar to young mice,

old mice fed coconut or corn oils showed a significant increase in liver tocopherol concentration when dietary tocopherol was increased. No significant rise in liver tocopherol concentration was observed in mice fed fish oil. There was a significant overall effect of fat on liver tocopherol concentration, which was more pronounced at the higher tocopherol concentrations. In young mice, there was a significant correlation between plasma and liver tocopherol concentrations (r = 0.82, 0.76 and 0.41 for mice fed coconut oil, corn oil and fish oil, respectively). Plasma and liver tocopherol in the old mice were not significantly correlated.

Tables 8 and 9 show the tocopherol concentration of kidneys in young and old mice, respectively. Old mice had similar or slightly higher tocopherol concentrations than young mice. A significant fat effect was observed in young and old mice only at 500 ppm dietary vitamin E. There was a significant increase of kidney vitamin E concentration in young and old mice fed corn and coconut oils with increased dietary tocopherol. No significant rise of kidney tocopherol concentration in young mice fed fish oil was observed (Table 8). In old mice, a significant increase in kidney vitamin E concentration was observed regardless of the type of fat; however, the increase was less in mice fed fish oil. The fat effect on tocopherol concentration in kidney was significant only when dietary tocopherol was 500 ppm, at which level mice fed fish and corn oils had lower kidney tocopherol concentrations than mice fed coconut oil (Tables 8 and 9). Plasma tocopherol level was correlated with kidney tocopherol in mice fed coconut oil (r = 0.79 in young mice; r = 0.72 in old mice). A weaker correlation was observed in mice fed corn oil (r = 0.46 for young mice; r = 0.44 for old mice). In mice fed fish oil, plasma tocopherol did not correlate with kidney levels (r = 0.13), although a weak correlation was observed in old mice (r = 0.51). The same situation held for correlations between plasma and lung tocopherol levels (r = 0.17 in young mice; r = 0.58 in old mice).

Tables 10 and 11 show the concentration of tocopherol in lungs of young and old mice, respectively. Tocopherol concentrations in lung were very close to those of kidney; the values for young and old mice were also similar, with the old mice having slightly lower concentrations. Similarly, a significant increase in lung tocopherol concentration was observed only in young mice fed coconut and corn oils supplemented with 100 or 500 ppm tocopherol. Mice fed fish oil had a significantly lower lung tocopherol concentration than mice fed coconut oil (Tables 10 and 11).

TABLE 8

Kidney Tocopherol Concentration in 3-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil, or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/g)	Corn oil (μg/g)	Fish oil (μg/g)	
30	11 ± 1 (n = 9)	9 ± 2 (n = 9)	11 ± 1 (n = 7)	NS
100	12 ± 1 (n = 10)	12 ± 1 (n = 8)	11 ± 1 (n = 10)	NS
500	23 ± 2[a] (n = 9)	16 ± 1[a,b] (n = 10)	12 ± 1[b] (n = 7)	$P < 0.002$
	$P < 0.0001$	$P < 0.004$	NS	

Mean ± SE. NS, not significant.

[a]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.

[b]Significantly different from mice fed coconut oil at P values indicated in the last column of each row.

TABLE 9

Kidney Tocopherol Concentration in 24-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/g)	Corn oil (μg/g)	Fish oil (μg/g)	
30	11 ± 2 (n = 9)	12 ± 1 (n = 8)	14 ± 1 (n = 10)	NS
100	22 ± 3[a] (n = 7)	18 ± 2[a] (n = 7)	17 ± 3 (n = 6)	NS
500	25 ± 3[a]	21 ± 2[a,b]	20 ± 4[a,b]	$P < 0.03$
	$P < 0.0001$	$P < 0.02$	$P < 0.04$	

Mean ± SE. NS, not significant.

[a]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.

[b]Significantly different from mice fed coconut oil at P values indicated in the last column of each row.

DISCUSSION

Even before the role of vitamin E in preventing nutritional muscular dystrophy was fully recognized, the ability of cod liver oil to precipitate this lesion, particularly in herbivores, was well established (12). Later, it was demonstrated that vitamin E can antagonize the tendency of cod liver oil to induce muscular dystrophy. MacKenzie et al. (13), however, showed that vitamin E could not prevent muscular dystrophy if administered orally in cod liver oil. This effect of cod liver oil was attributed to its content of highly unsaturated fatty acids. Furthermore, since aging is associated with increased lipid peroxidation, and senescent rodents may have a higher requirement for tocopherol (16,17), we studied the effect of a low level (5%), short-term (6-wk) diet of fish oil, corn oil or coconut oil on the vitamin E status of young and old mice fed adequate or high levels of tocopherol.

The results indicate that young mice fed fish oil have significantly lower plasma tocopherol levels than those fed corn and coconut oils at all three levels of dietary tocopherol tested (30, 100 and 500 ppm). Mice fed corn oil tended to have lower tocopherol levels than mice fed coconut oil, but differences were significant only at 500 ppm dietary tocopherol. Mice fed coconut and corn oils showed significant increases in plasma tocopherol as dietary tocopherol increased. No significant increase in plasma tocopherol concentration was observed in mice fed fish oil. The lower plasma tocopherol level and lack of response to an increase in dietary tocopherol in mice fed fish oil was not due to oxidative loss of tocopherol in the

TABLE 10

Lung Tocopherol Concentration in 24-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/g)	Corn oil (μg/g)	Fish oil (μg/g)	
30	9 ± 1 (n = 6)	9 ± 4 (n = 2)	11 ± 0.4 (n = 2)	NS
100	16 ± 1[a] (n = 6)	16 ± 2 (n = 4)	12 ± 1[b] (n = 8)	NS
500	23 ± 2[a] (n = 10)	18 ± 3[b] (n = 9)	NA	$P < 0.009$
	$P < 0.0008$	$P < 0.01$	NS	

Mean ± SE. NS, not significant. NA, data not available.
[a]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.
[b]Significantly different from mice fed coconut oil at P values indicated in the last column of each row.

TABLE 11

Lung Tocopherol Concentration in 3-Month-Old C57BL/6Nia Mice Fed Coconut Oil, Corn Oil or Fish Oil Diets Supplemented with Different Levels of Vitamin E

Vitamin E level (ppm)	Coconut oil (μg/g)	Corn oil (μg/g)	Fish oil (μg/g)	
30	11 ± 1 (n = 5)	9 ± 1 (n = 2)	11 ± 1 (n = 3)	NS
100	16 ± 1 (n = 6)	10 ± 2[a] (n = 4)	11 ± 2[a] (n = 5)	$P < 0.053$
500	18 ± 1[b] (n = 6)	22 ± 1[b] (n = 4)	NA	NS
	$P < 0.0002$	$P < 0.0001$	NS	

Mean ± SE. NS, not significant. NA, data not available.
[a]Significantly different from mice fed coconut oil at P values indicated in the last column of each row.
[b]Significantly different from mice fed 30 ppm vitamin E at P values indicated in the last row of each column.

diet (Table 3). The lower plasma tocopherol level in mice fed fish oil can be due either to an interaction of fish oil and tocopherol at the gut level, as suggested by others (13,18,19), or to enhanced postabsorptive utilization of tocopherol compared to mice fed coconut and corn oils. Further studies are needed to clarify the mechanism of action.

In mice fed corn oil, the liver tocopherol concentration was lower than that of mice fed coconut oil and higher than that of those fed fish oil, which correlates well with the differences in degree of unsaturation between corn and fish oils. It is worth noting that only mice fed fish oil supplemented with 500 ppm vitamin E had plasma and liver tocopherol levels comparable to those fed coconut oil with 30 ppm vitamin E. This agrees with previous reports (13) that concurrent administration of fish oil and tocopherol did not alleviate signs of tocopherol deficiency. Old mice had lower plasma tocopherol levels than young mice, except those fed fish oil (see also 16). The higher plasma tocopherol level of old mice compared to that of young mice fed fish oil may be due to the larger pool of available tocopherol in old mice.

The effect of fat type and tocopherol supplementation on liver tocopherol concentration was similar in young and old mice. Mice fed fish and corn oils had lower tocopherol concentrations than those fed coconut oil. Increasing tocopherol concentration in mice fed fish oil did not increase liver tocopherol concentration. No age effect was apparent when old mice were compared to young mice fed fish oil; when plasma and liver tocopherol concentrations in old mice fed fish oil were compared to those of young mice fed other fats, a significant age effect was observed. The lower plasma tocopherol concentration in the aged mice may be due to decreased absorption and/or increased uptake and utilization of vitamin E by other tissues. This mechanism could be determined by measuring the postabsorption level of α-tocopherol in the lymphatic duct and unabsorbed tocopherol in the feces. Since kidney and lung tocopherol concentrations in old mice were equal to or slightly higher than those in young mice, the lower plasma concentration may be due to decreased absorption. If lower plasma and liver tocopherol content in old mice were due to increased catabolism, the age effect should have been exaggerated rather than abolished in mice fed fish oil.

The dietary fat effect on tocopherol concentrations of kidney and lung was significant only at the higher dietary vitamin E concentrations. This was in contrast to plasma and liver tocopherol concentrations, but in agreement with other reports (21,22). Studies in guinea pigs and rats (21,22) indicate that the depletion of tocopherol from plasma and liver is rapid relative to other organs. Therefore, a 6-wk dietary treatment might not have been long enough to reduce the tocopherol content of kidney and lung in mice fed fish oil. No significant rise in kidney and lung concentrations of tocopherol was observed in young mice fed fish oil, reflecting the observations in plasma and liver.

In conclusion, even after a short-term dietary regimen, animals fed fish oil maintain a lower tocopherol status than those fed corn or coconut oils. This effect should be taken into consideration when designing and interpreting experiments where the biological effects of fish oil are compared to those of other oils. Furthermore, plasma and liver levels of tocopherol are influenced by age in mice, whereas kidney and lung tocopherol do not appear to be altered by age when the diet contains 30 ppm or more vitamin E. The consequences of inadequate vitamin E intake by young and old individuals consuming different dietary fats should be examined.

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Phosphatidylcholine as the Choline Donor in Sphingomyelin Synthesis[1]

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Sphingomyelin synthesis was studied in cultured Novikoff rat hepatoma cells by following transfer of [^{14}C]choline label into sphingomyelin (SPH). The study was facilitated by the fact that prelabeling of the cells with [*methyl*-^{14}C]choline resulted in rapid accumulation of essentially all the label (~95%) in phosphatidylcholine (PC). The redistribution of PC label during a 15-hr chase was dependent upon the extracellular choline concentration. Under conditions of free choline diffusion (500 μM choline), loss of label from PC was most pronounced, and the percentage of total radioactivity that became trapped in the extracellular water-soluble choline pool was an order of magnitude greater than in low choline medium (27 μM choline). Despite the significant loss of water-soluble label from the cells in high choline medium, SPH labeling proceeded at essentially the same rate at either choline concentration. During the label chase in 500 μM choline, the specific radioactivity of PC decreased, but the specific radioactivity of SPH continued to increase for 9–12 hr until it reached the specific radioactivity of PC. In the presence of 300 μM neophenoxine (NPO), transfer of label from PC into SPH was stimulated. NPO also decreased the specific radioactivity of PC to about the same extent as that of SPH was increased. Because transfer of choline label from PC to SPH was not affected by loss or dilution of water-soluble precursors, and because the specific radioactivity of PC and SPH, in the absence or presence of NPO, responded in a characteristic precursor product fashion, we conclude that sphingomyelin synthesis in Novikoff cells circumvents the water-soluble choline pool and that phosphatidylcholine serves as the immediate choline source. All our data support the phosphatidylcholine:ceramide cholinephosphotransferase pathway of sphingomyelin synthesis.
Lipids 22, 351–357 (1987).

Sphingomyelin (SPH) synthesis has been studied for almost three decades, with several pathways being established, and challenged thereafter. Early in vitro studies with particulate fractions from chicken liver hinted that SPH is synthesized by phosphocholine transfer from cytidine 5′-diphosphocholine (CDPcholine) to ceramide (2–4) quite in analogy to de novo phosphatidylcholine (PC) synthesis (5). In vivo experiments in rat brain showed that ceramide serves as SPH precursor (6). Evidence also emerged that sphingosylphosphocholine can be acylated with acyl-CoA by rat brain microsomes (7,8), yet the biosynthesis of sphingosylphosphocholine itself (9) remained uncertain (10,11).

Studies with SV-40 transformed mouse fibroblasts provided a first clue that the phosphocholine donor in SPH synthesis may be not CDPcholine but phosphatidylcholine (12–15), and ceramide was shown to serve as phosphocholine acceptor in this reaction (16). The PC:ceramide cholinephosphotransferase pathway was also demonstrated in lyophilized microsomes from mouse liver (17); however, in rat liver microsomes, CDPcholine and not PC appeared to be the phosphocholine donor (10). More recent studies on the cellular and subcellular level have provided additional evidence for the phosphatidylcholine:ceramide cholinephosphotransferase pathway in sphingomyelin synthesis (11, 18–22).

In the present study, we have examined the routes of SPH synthesis in cultured Novikoff rat hepatoma cells. Three characteristics of these cells proved particularly useful. First, we observed that when Novikoff cells are pulsed with [^{14}C]choline in spent medium, they rapidly incorporate essentially all the label into PC (~95%). This simplified following the flux of the label during the chase and facilitated interpretation of label chase kinetics. Second, Novikoff cells are known to take up choline by two different mechanisms (23). At low extracellular choline levels (<20 μM), active transport prevails, and the rate of choline transport is actually the rate-limiting step in choline phosphorylation. At higher choline concentrations, simple diffusion at a rate three times higher than active transport is the principal mode. These distinct modes of choline uptake permitted us to modulate the water-soluble choline pool and to evaluate its contribution to SPH synthesis. Third, we found that neophenoxine (NPO; 24) stimulates SPH synthesis in Novikoff cells at the expense of PC labeling. This made it possible to also modulate the flux of label through the lipid-associated choline pool and to assess the effect of such modulation on sphingomyelin synthesis.

EXPERIMENTAL PROCEDURES

Materials. S_{210} medium was obtained from Grand Island Biological Co. (Gibco, Grand Island, New York). S_{210} consists of Swim's 67G medium supplemented with succinic acid (75 mg/l) and sodium succinate (100 mg/l). Choline chloride was from Aldrich Chemical Co. (Milwaukee, Wisconsin) and [*methyl*-^{14}C]choline chloride from New England Nuclear (Boston, Massachusetts). Lysophosphatidylcholine (lysoPC) was prepared from egg yolk PC by phospholipase A_2 hydrolysis (25) using *Ophiophagus hannah* venom (Miami Serpentarium, Miami, Florida). NPO, *p*-chlorophenoxyethyl *N,N*-dimethylaminoethyl ether, was synthesized as we described previously (24).

Cell system. Novikoff rat hepatoma cells, subline N1S1-67 (26), were grown in shaker culture (110 rpm) at 37 C in S_{210} medium (pH 7.4) supplemented with meat peptone (2 g/l), penicillin (10^5 units/l), streptomycin (100 mg/l) and 5% newborn calf serum (K.C. Biologicals, Kansas City, Missouri). Cells were subcultured twice per week from a starting density of 3.5×10^5 cells/ml using fresh medium. Cell growth was monitored by cell counting and by following the rate of [^{3}H]thymidine incorporation. Thymidine incorporation was measured by incubating 2-ml samples of cell suspension with 0.5 μCi of

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[^{3}H]thymidine (New England Nuclear; sp act 2 Ci/mmol) for 1 hr (27); the cells were washed three times with 2 ml of phosphate-buffered saline, and cell-associated and/or TCA precipitate-associated radioactivity (cpm/mg protein) was determined by scintillation counting. Protein was assayed according to Lowry et al. (28). Cell viability was routinely monitored by the trypan blue exclusion test. Cells used for experiments were taken from their logarithmic phase of growth (48 hr); in all experiments described, cell viability exceeded 95%.

Label chase in low and high choline media. Two-day-old Novikoff hepatoma cell cultures, averaging 2×10^6 cells/ml, were incubated, without changing the medium, with 1.5 μCi of [*methyl*-^{14}C]choline chloride (sp act 53.5 mCi/mmol) per 10^7 cells for 1.5 hr under standard growth conditions. After pulse labeling, the cells were washed twice with fresh growth medium and then were resuspended at a density of 1.5×10^6 cells/ml in one of three chase media: (a) the basal growth medium containing 27 μM choline (low choline medium); (b) the basal growth medium supplemented with choline chloride to a final concentration of 500 μM choline (high choline medium); and (c) the high choline medium (500 μM choline) supplemented with 300 μM NPO. Choline in the basal growth medium originated from choline bitartrate (15 μM) present in S_{210} medium and the choline in pluronic (7 μM), calf serum (5 μM), and meat peptone (<0.1 μM). Choline was assayed according to Gibson et al. (29). Chase incubations were carried out on triplicate cultures under standard growth conditions. At given intervals (3, 6, 9, 12 and 15 hr) in the 15-hr chase, 2.5-ml aliquots were removed from each culture and the samples were quickly chilled in ice. The cells were sedimented at 500 × g for 5 min, washed once with cold growth medium and analyzed. The supernatants were combined and kept frozen until analyzed.

Effect of choline concentration on [^{14}C]choline incorporation. Two-day-old cells were washed with fresh growth medium and then resuspended at 1.5×10^6 cells/ml in either low choline (27 μM) or high choline (500 μM) medium, each containing 54,000 cpm/ml of [*methyl*-^{14}C]-choline. The cells were incubated for 15 hr under standard growth conditions, and 2.5-ml aliquots were taken at given intervals. The cells were sedimented at 500 × g for 5 min and washed with cold growth medium, and phospholipid-associated label and water-soluble label in the cells were measured.

Analyses. Cells and culture media were extracted according to Bligh and Dyer (30). LysoPC was usually added to the extract to serve as carrier. Lipids were fractionated by thin-layer (0.5 mm) chromatography (TLC) on Silica Gel H (Merck, Darmstadt, West Germany) using chloroform/methanol/water (65:35:8, v/v/v) as developing solvent (solvent A). Fractions were made visible by brief exposure to iodine vapors. Individual fractions were assigned by comparing their migration rates with those of standards. In analytical TLC, choline phospholipids were also identified by spraying with a choline-specific molybdenum reagent (31). In preparative TLC (solvent A), bands corresponding to PC (Rf 0.43), SPH (Rf 0.33) and lysoPC (Rf 0.22) were scraped off, and the phospholipids were recovered by three successive elutions with chloroform/methanol/water (50:40:10, v/v/v, once; then 60:30:5, v/v/v, twice). Rechromatography of the PC and the SPH fractions on Silica Gel H layers, with chloroform/methanol/conc. aqueous ammonia (65:25:5, v/v/v; solvent B) as developing solvent (32) showed that in each case more than 90% of the radioactivity was associated with PC (Rf 0.27) and SPH (Rf 0.13). The SPH fraction proved resistant to hydrolysis in 1 N sodium hydroxide (33).

The water-soluble components of the upper Bligh-Dyer phase from cells and media were fractionated by TLC on Silica Gel H in the presence of carriers using 1-propanol/conc. aqueous ammonia (1:1, v/v) as developing solvent (15). Fractions were made visible by exposure to iodine vapors. The fractions comigrating with choline (Rf 0.04), phosphocholine (Rf 0.17), glycerophosphocholine (Rf 0.31) and CDPcholine (Rf 0.38) were isolated, and their radioactivities were measured after elution with methanol/water (1:2, v/v).

Radioactivity was counted on aliquots of the samples (400–20,000 cpm) using 10 ml of scintillation fluid. Scintillation fluid was prepared from 12 g of Omnifluor (New England Nuclear), Triton X-100 (1 l) and toluene (2 l). Phospholipid radioactivities given are those determined after TLC fractionation using solvent A. The specific radioactivity of PC and SPH was determined after repurification of the samples using solvent B. Phospholipid phosphorus was measured according to Bartlett (34).

RESULTS

The study was designed to compare the participation of the water-soluble and the lipid-associated choline pools in the transfer of [^{14}C]choline to SPH. Short-term exposure (90 min) of two-day-old Novikoff cells to [^{14}C]choline in spent medium resulted in the rapid accumulation of 92 to 98% of the total cell label in PC (Fig. 1). Only small amounts of label were initially associated with SPH (<3%) or water-soluble choline components.

Label chase in low and high choline media. Label was chased over 15-hr periods in either normal growth medium containing 27 μM choline or high choline medium containing 500 μM choline. Cells chased in either medium remained morphologically identical; showed identical growth rates as judged by cell count, protein content and rate of [^{3}H]thymidine incorporation (27); and maintained identical rates of phospholipid synthesis. During the entire chase, cell numbers typically increased 1.8-fold, and phospholipid content increased 2.5-fold in either culture.

Figure 1 compares the redistribution of label originally concentrated in PC within the 15-hr chase at low and high choline levels. It is apparent that in 27 μM choline there was less loss of label from PC (from 95 to 80% of total label) than in 500 μM choline (from 95 to 55%). Under conditions of free choline diffusion, loss of PC label was quite pronounced.

Figure 1 furthermore shows that at the low choline concentration, the proportion of water-soluble label in the medium remained rather constant (2–3%). At the high choline concentration, by contrast, the substantial loss of label from cell PC was reflected in an increased release of water-soluble label into the medium. The percentage of total radioactivity that finally appeared in the water-soluble choline pool of the high choline medium was about 10 times greater (23% of total) than that released into

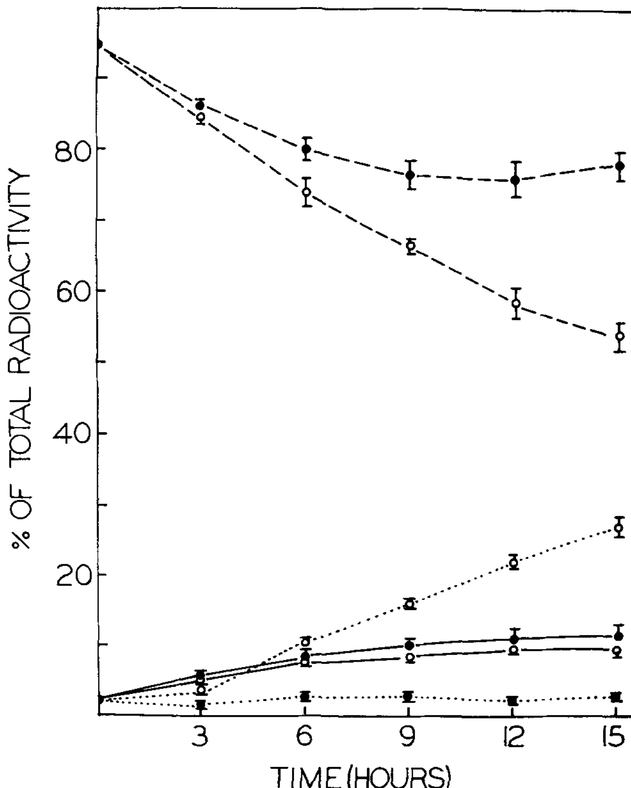

FIG. 1. Redistribution of [^{14}C]choline label in cultured Novikoff hepatoma cells during label chase in low and high choline media. Cells (2.25 × 10^{7} cells/15 ml) were prelabeled with [*methyl*-^{14}C]choline for 1.5 hr, and the label was chased for 15 hr in media containing either 27 μM (●) or 500 μM (○) choline. Redistribution of label between cell PC (— —), cell SPH(——) and the water-soluble components released into the medium (.....) is shown as percentage of total label recovered at various intervals from 2.5-ml aliquots of culture (1.3–1.7 × 10^{5} cpm). The data represent means of three triplicate experiments ± standard errors (n = 9).

the low choline medium (Fig. 1). TLC showed that about 90% of the label in high choline medium was actually associated with free choline. Progressive accumulation of [^{14}C]choline in the high choline medium suggests that label entering the medium was trapped there. Obviously, labeled choline could not effectively re-enter the cells and participate in phospholipid synthesis because of dilution with the very large excess of unlabeled choline in the medium.

The most significant finding that emerged from these experiments was that even though cells chased for 15 hr in high choline medium irretrievably lost most of the water-soluble label released from PC into the medium, they transferred label to SPH with essentially the same efficiency as did low choline cells. The radioactivity associated with cell SPH increased essentially at the same rate and reached about the same level (14% of total label) at either choline concentration (Fig. 1). Because transfer of choline label to SPH was not affected by loss or dilution of water-soluble precursors, these data strongly suggested that SPH synthesis in Novikoff cells circumvents the water-soluble choline pool and instead utilizes lipid-associated choline as precursor.

To assess the contribution of lipid-associated and water-soluble choline precursors to SPH synthesis in a more quantitative fashion, we compare in Table 1 label chase data of a representative experiment similar to the one described above (Fig. 1). Table 1 shows that initial PC labeling was high (153,000 cpm) and that only small amounts of radioactivity were initially associated with lysoPC (2,500 cpm), SPH (4,500 cpm) and the water-soluble choline components (7,500 cpm) of the cells. Throughout the chase, lysoPC labeling remained constant in low choline medium, but decreased in high choline medium (Table 1; NPO data are discussed below). Lipid-associated radioactivity released into the medium (largely as lysoPC) was quite independent of the extracellular choline concentration. The amount of water-soluble label in the cells grown in either medium was substantial (7,500–13,000 cpm) and was shown to be associated with phosphocholine (58–63%), glycerophosphocholine (28–33%), CDPcholine (5–9%) and free choline (1–3%). There was little change in this percentage distribution in the course of the chase. Total water-soluble label increased significantly during the chase in high choline cells (from 7,500 to 13,000 cpm). The relatively low level of water-soluble label (8,500 cpm) that remained in the cells after 15 hr in low choline medium is likely to reflect recycling of water-soluble label into choline phospholipids because of a general depletion of water-soluble precursors. This explanation is consistent with the fact that after 15 hr the amount of water-soluble label in the medium was also quite low (4,000 cpm). After 15 hr in high choline medium, by contrast, water-soluble label released into the medium reached a level about 10 times higher (41,000 cpm) than in low choline medium. The latter vividly illustrates the susceptibility of the Novikoff cell system to choline modulation.

Table 1 also shows that during the 15-hr chase in low choline medum, PC suffered only a relatively moderate loss of label (from 153,000 to 127,500 cpm); however, in high choline medium, loss of label from PC was substantial (from 153,000 to 91,000 cpm). The cells chased in 500 μM choline lost almost 2.5 times as much label (62,000 cpm) from PC as those chased in 27 μM choline (25,500 cpm); most of the difference (36,500 cpm) was accounted for by the increase of label in the water-soluble choline pool of the medium (41,000 cpm). During the same time, also, SPH labeling increased from 4,500 to 18,500 cpm in high choline cells and to 23,500 cpm in low choline cells. If one would presuppose that PC is the precursor of SPH, the somewhat lower level of SPH labeling in high choline cells could simply reflect the lower levels of PC label available in these cells. This is also consistent with the observation that the ratios of label associated with cell SPH vs cell PC (SPH/PC ratios) at 9 hr were essentially identical for cells grown in low (0.16) or high (0.15) choline medium, and the same held true at 15 hr (0.18 and 0.20, respectively). We therefore can conclude that SPH labeling is closely linked to PC labeling and is independent of the size of the water-soluble choline pool.

To further scrutinize the apparent metabolic relationship between PC and SPH, we followed their specific radioactivities during the label chase in high choline medium (Fig. 2; NPO data are discussed below). The plot illustrates that the specific radioactivity of PC declined throughout the entire 15-hr chase. By contrast, the specific radioactivity of SPH increased and then peaked at 9–12 hr when the specific radioactivity of SPH equaled the specific radioactivity of PC. This specific radioactivity pattern corresponds to the mathematical description of

TABLE 1

Redistribution of [^{14}C]Choline Label in Novikoff Hepatoma Cells During Label Chase in Low and High Choline Media and the Effect of Neophenoxine Supplementation[a]

	Distribution of radioactivity, cpm (% of total)						
		9 hr			15 hr		
Fractions	0 hr	27 μM choline	500 μM choline	500 μM choline 300 μM NPO	27 μM choline	500 μM choline	500 μM choline 300 μM NPO
Cells							
Lysophosphatidylcholine	2,336 (1.3)	2,429 (1.4)	1,437 (0.9)	2,533 (1.5)	2,279 (1.3)	1,140 (0.7)	2,528 (1.5)
Sphingomyelin	4,440 (2.7)	20,465 (11.9)	16,140 (9.6)	23,420 (14.2)	23,390 (13.6)	18,580 (10.9)	23,615 (13.9)
Phosphatidylcholine	153,286 (91.5)	127,345 (74.2)	110,665 (65.5)	88,015 (53.6)	127,700 (74.3)	91,235 (53.8)	75,500 (44.4)
Water-soluble[b]	7,473 (4.5)	12,390 (7.2)	10,395 (6.2)	13,458 (8.2)	8,470 (4.9)	12,880 (7.6)	16,905 (10.0)
Culture medium							
Phospholipids	—	4,875 (2.9)	4,282 (2.5)	10,943 (6.7)	6,123 (3.6)	4,801 (2.8)	12,340 (7.3)
Water-soluble	—	4,092 (2.4)	25,916 (15.3)	25,872 (15.8)	3,960 (2.3)	40,964 (24.2)	38,940 (22.9)
Total radioactivity	167,535 (100)	171,596 (100)	168,835 (100)	164,261 (100)	171,922 (100)	169,600 (100)	169,828 (100)

[a]Novikoff cells (2.25 × 10^7 cells/15 ml) were prelabeled with [^{14}C]choline for 1.5 hr and the label was chased for up to 15 hr in media containing 27 μM choline, or 500 μM choline in the absence or presence of 300 μM neophenoxine. The distribution of radioactivity (cpm) at various time points was determined on 2.5-ml aliquots of culture. Typical counting efficiency was >90%. Values given are averages of three incubations; average differences from the mean were less than ±5%.

[b]Water-soluble choline label in the cells was associated with phosphocholine (58–63%), glycerophosphocholine (28–33%), CDPcholine (5–9%) and free choline (1–3%).

a direct precursor-product relationship (35) and serves as additional, strong evidence that PC is the direct precursor of SPH.

Effect of choline concentration on [^{14}C]choline incorporation. The label chase experiments described above demonstrated that the redistribution of PC label was very dependent on the extracellular choline concentration. To ascertain that cells chased in high choline medium indeed took up enough unlabeled choline to account for the apparent dilution and subsequent depletion of intracellular, water-soluble choline label released from PC, choline uptake and intracellular redistribution of phospholipid and water-soluble label was followed at both choline concentrations. The experimental protocol was essentially that of the pulse label studies, except that the cells were carried through the "prelabeling" phase in the absence of radiolabel and through the "15-hr chase" phase in the presence of identical amounts (dpm) of radiolabel in 27 μM or in 500 μM choline (for details, see Experimental Procedures).

A comparison of [^{14}C]choline incorporation into the phospholipids of Novikoff cells grown in low (Fig. 3A) or high (Fig. 3B) choline medium shows that final incorporation of label into total choline phospholipids of low choline cells (118,000 cpm) was about 18.2-fold greater than incorporation of label into choline phospholipids of high choline cells (6,500 cpm). This ratio of choline phospholipid label in low vs high choline cells is essentially the same as the ratio (18.5:1) of the respective specific radioactivities of the extracellular choline provided in the two media. This is what one would expect of cells having equal rates of phospholipid synthesis but precursor pools of different specific radioactivities. These data furthermore imply that complete equilibration between intracellular and extracellular water-soluble choline label must have been attained within the time frame of the present experiment.

During the 15-hr experiment, choline labeling of the intracellular water-soluble pool followed a pattern quite different from that of choline phospholipids. Whereas cells grown in 500 μM choline kept accumulating water-soluble label at an essentially constant rate over the entire 15 hr growth period (Fig. 3B), the water-soluble label in cells grown in 27 μM choline (Fig. 3A) peaked at about 9 hr and then showed a rapid decline, reflecting choline depletion of the cultures.

Figure 3C depicts changes in the ratios of water-soluble vs phospholipid-associated cell label during the 15-hr growth phase in the two media. The data demonstrate that at the end of the experiment, the ratio of water-soluble vs phospholipid-associated label was about 14 times greater in high choline cells than it was in low choline cells. As we had shown that the rates of phospholipid synthesis were identical in both cells, the increased ratios of water-soluble vs phospholipid-associated label in high choline cells do directly reflect increased choline uptake.

Stimulation of SPH synthesis by NPO. We had previously shown (24,36) that hypolipidemic drugs of the clofibrate type, as well as their structural analogs

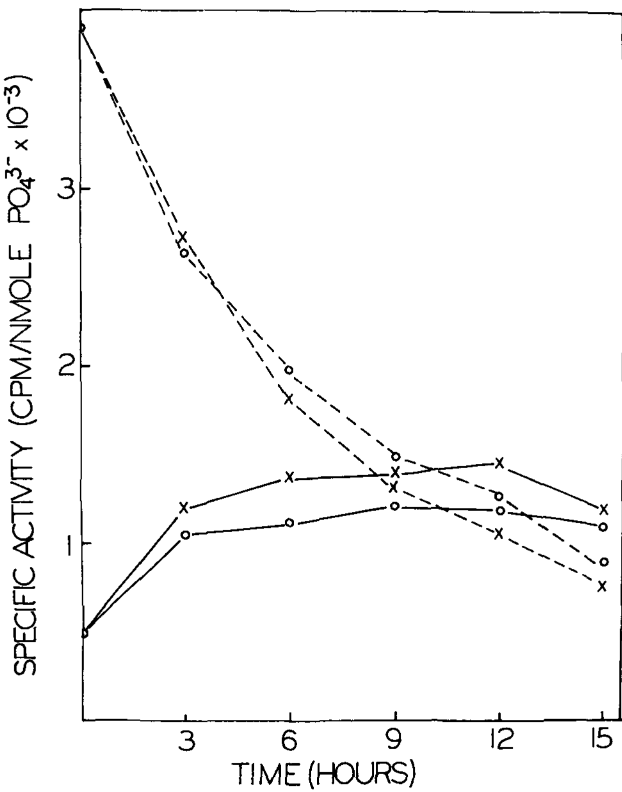

FIG. 2. Specific radioactivity of phosphatidylcholine and sphingomyelin in Novikoff cells during label chase in high choline medium. Cells were prelabeled with [*methyl*-^{14}C]choline, and the label was chased for up to 15 hr in media containing 500 μM choline (O) or 500 μM choline plus 300 μM neophenoxine (X). The specific radioactivity of PC (– – –) and SPH (——) was determined on pooled triplicate samples (experiments Table 1) that had been repurified by TLC (developing solvent B).

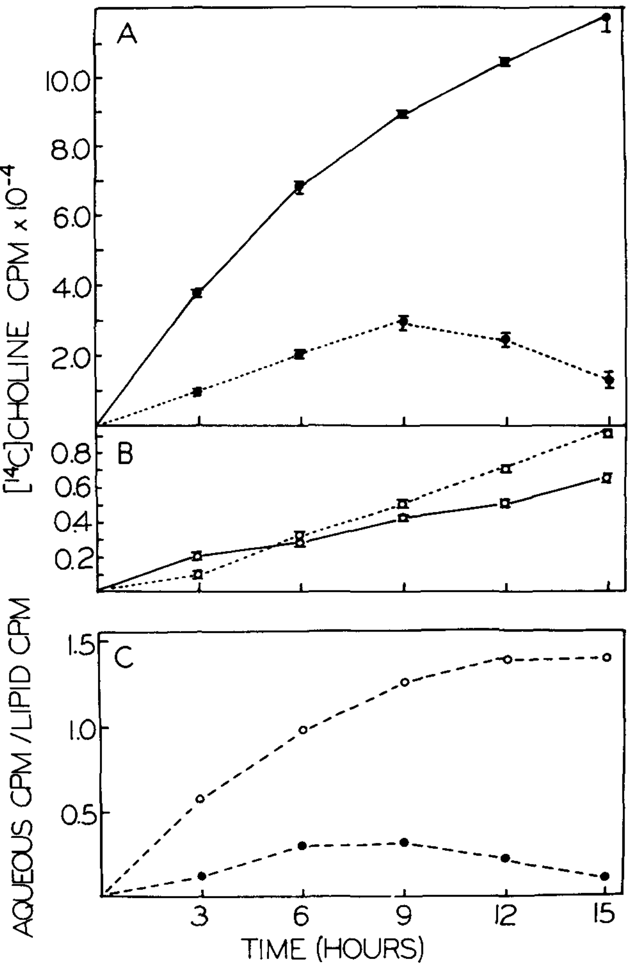

FIG. 3. Effect of choline concentration on [*methyl*-^{14}C]choline incorporation into the phospholipid and water-soluble cholines of Novikoff cells. Cells (2.25 × 10^7 cells/15 ml) were incubated for up to 15 hr with [^{14}C]choline (54,000 cpm/ml) in media containing either 27 μM (●) or 500 μM (O) choline. The distribution of label between the phospholipid (——) and water-soluble (.....) fractions of the cells from 2.5-ml aliquots of culture was followed in low (A) and high (B) choline medium. Changes in the ratios of water-soluble vs lipid-associated cell label (— —) at the two choline concentrations are shown in C. The data represent means ± deviations (n = 3).

centrophenoxine and neophenoxine, inhibit both enzymes of microsomal PC synthesis, namely CDPcholine:diacylglycerol cholinephosphotransferase and particularly acyl-CoA:lysolecithin acyltransferase (LLAT). In the present study, NPO was used to modulate the flux of label through the lipid-associated choline pool and to follow the effect of such modulation on SPH synthesis.

Table 1 shows the effect of 300 μM NPO during the 15-hr label chase in high choline (500 μM) medium. At this concentration, NPO did not affect cell growth. However, NPO led to a substantial loss in PC labeling (15,500 cpm). NPO, a potent inhibitor of lysoPC reacylation (24), also caused, as expected, an increase in cell lysoPC labeling (from 1,000 to 2,500 cpm). Moreover, there was a substantial increase (7,500 cpm) in the amount of phospholipid label found in the medium, and we could show that essentially all additional phospholipid label secreted by the cells was associated with lysoPC. The water-soluble pools, by contrast, were only little affected by NPO. However, NPO caused a significant stimulation of SPH synthesis (by 5,000 to 7,000 cpm) at the expense of PC labeling (Table 1).

Stimulated flux of label from PC to SPH due to NPO also became apparent when the specific radioactivities of PC and SPH were followed throughout the 15-hr chase. Figure 2 illustrates that the decrease in the specific radioactivity of PC due to NPO was mirrored in a concomitant increase of similar magnitude in the specific radioactivity of SPH. In the presence of NPO, the specific radioactivity of PC and SPH again showed the familiar precursor-product pattern.

Inhibition of SPH synthesis by p-chloromercuribenzoate (p-CMB). As a sulfhydryl reagent that is not readily internalized by cells, *p*-CMB has been used to inhibit enzymes located near the cell surface (37). Our inhibition studies with *p*-CMB were carried out following the protocol of the label chase experiments in low choline medium, except that *p*-CMB was added at time 0 and the chase was continued for only 5 hr to minimize the toxic effects of the agent. We observed that 0.025 mM *p*-CMB did not affect SPH labeling, but that 0.5 mM *p*-CMB proved highly toxic to the cells. At 0.10–0.15 mM, *p*-CMB did not cause cell death (viability > 90%). However, at these *p*-CMB concentrations transfer of label from PC to SPH was completely inhibited.

DISCUSSION

The discovery of the pathway of de novo phosphatidylcholine synthesis from diacylglycerol and CDPcholine 30 years ago (5) ranks as one of the milestones in lipid biochemistry. In the wake of this breakthrough, a route of SPH synthesis from ceramide and CDPcholine appeared equally attractive. Early in vitro studies with short chain (*N*-acetyl), isomeric ceramides and with CDPcholine as water-soluble phosphocholine donor were indeed encouraging (2,3). Yet questions remained, because the CDPcholine:ceramide cholinephosphotransferase required ceramides with an *N*-acetyl function instead of the natural, long chain *N*-acyl group, and it also showed a pronounced preference for *threo* ceramides, although natural sphingolipids possess the *erythro* configuration (38–40). Numerous attempts were made over the years to correct or rationalize these inconsistencies (4,41–43), and it was only a few years ago that reasonable evidence emerged that rat liver microsomes may be able to transfer phosphocholine from CDPcholine to *N*-palmitoyl-*erythro*-sphingenine (10).

For some time, the possibility was also considered that SPH may be synthesized by acylation of sphingosylphosphocholine. Studies with rat brain microsomes suggested that the acyl-CoA transacylase preferred *erythro*-sphingosylphosphocholine over its *threo* isomer (7,8); however, this pathway could not be confirmed in rat liver microsomes (10). Also, the synthesis of sphingosylphosphocholine from sphingenine, which was first observed in a particulate fraction from chicken liver (9), could not be reproduced in other systems (10,11).

In vivo experiments in rat brain had early shown that ceramide is readily converted to SPH (6). Also, the synthesis of ceramide from sphingenine and acyl-CoA became firmly established (10,44,45). A first indication that SPH synthesis from ceramide may utilize not water-soluble CDPcholine but the phospholipid pool as phosphocholine source emerged from studies with cultured SV-40 transformed mouse fibroblasts. By following the label chase kinetics of phosphate incorporation into individual phospholipid classes of these cells, it became apparent that phospholipid labeling generally reached constant rates within one hr, but that in the case of SPH a 6-hr lag phase did occur (12,14). Pulse labeling studies with [^{32}P]phosphate and [^{3}H]choline demonstrated that the ^{3}H/^{32}P ratio of PC and SPH remained quite constant throughout the chase and that PC labeling peaked at 3 hr, whereas SPH labeling increased for 20 hr (13), suggesting that PC and SPH use different precursor pools. Results of experiments with cell-free systems from SV-40 transformed mouse fibroblasts furthermore indicated that SPH labeling is not directly affected by choline, phosphocholine or CDPcholine, and that ceramide serves as phosphocholine acceptor in SPH synthesis (15,16). At about the same time, Ullman and Radin (17) demonstrated that lyophilized mouse liver microsomes can catalyze SPH synthesis from *erythro*-ceramide and that PC, but not CDPcholine, can serve as phosphocholine donor (18). However, rat liver microsomes were not able to utilize PC as phosphocholine donor, but CDPcholine was effective (10).

Evidence for a PC-dependent but CDPcholine-independent mode of SPH synthesis has also emerged from studies on a temperature-sensitive mutant of Chinese hamster ovary cells with a primary defect in CDPcholine synthetase activity (46). This mutant, when shifted to the restrictive temperature (40 C), showed an immediate decline in CDPcholine and PC labeling, where SPH synthesis remained stimulated for another 20 hr (47).

Labeling studies with cultured baby hamster kidney (BHK)-21 cells also provided rather strong evidence that CDPcholine could be excluded as the immediate source of the phosphocholine moiety of SPH and that PC was likely the in vivo phosphocholine donor (21). These conclusions were drawn by comparing the kinetics of [*methyl*-^{3}H]choline incorporation into CDPcholine and SPH and by following the transfer of [*methyl*-^{3}H]methionine-derived PC label to SPH. In the former experiment the primary evidence was that labeling of phosphocholine and CDPcholine rapidly declined during the chase while PC synthesis increased at a somewhat reduced rate during the first 2 hr and then declined; however, SPH synthesis proceeded at a nearly linear rate for at least 6 hr into the chase. The methionine labeling studies suggested a flux of label from PC to SPH without substantial CDPcholine labeling. However, the label chase experiments in the BHK system were somewhat hampered by the fact that at the onset of the [^{3}H]choline chase, nearly twice as much label was associated with phosphocholine as with phosphatidylcholine. It is also well known that the PC produced by the methylation pathway usually constitutes only a very small portion of total PC synthesis (48,49). This would still leave the possibility that parallel mechanisms of SPH synthesis do exist, with one pathway being dependent on the PC formed by the methylation route.

The experimental model we had on hand for the present study has clear advantages over those previously used. Prelabeling of cultured Novikoff rat hepatoma cells with [*methyl*-^{14}C]choline in spent medium resulted in the rapid accumulation of essentially all the label ($\sim$95%) in PC. This greatly facilitated following the flux of label during the chase. At 500 μM choline under conditions of free choline diffusion, loss of label from PC was much more pronounced than at 27 μM choline. In fact, the amount of water-soluble choline label trapped in high choline medium was an order of magnitude greater than in low choline medium. Yet despite the 10-fold dilution of water-soluble choline precursors in cells grown in high choline medium, SPH synthesis proceeded at essentially the same rate at both choline concentrations (Fig. 1 and Table 1). Because transfer of choline label from PC to SPH was not affected by loss or dilution of water-soluble precursors, we concluded that SPH synthesis in Novikoff hepatoma cells circumvents the water-soluble choline pool and that PC is the likely choline source for SPH synthesis.

Direct transfer of choline label from PC to SPH is also consistent with the changes in specific radioactivities that we observed for PC and SPH during the 15-hr chase. In a typical precursor-product fashion, the specific radioactivity of PC decreased while the specific radioactivity of SPH increased until SPH activity equaled PC activity (Fig. 2).

We furthermore found that NPO stimulated the transfer of label from PC to SPH. This made it possible to also modulate the flux of label through the lipid-associated choline pool and to follow specific radioactivity and precursor-product patterns under NPO-modulated conditions. The

mechanism by which NPO stimulates SPH synthesis from PC is presently not known; however, it is intriguing to note that certain glucocorticoids, such as dexamethasone, have also been shown to stimulate lysoPC and SPH synthesis in HeLa S3G cells (50) and to enhance SPH synthesis in 3T3-L1 fibroblasts (51). In the present context, the effect of NPO on PC and SPH labeling in Novikoff cells reemphasizes the close metabolic relationship between these two choline phospholipids.

It is well established that little SPH is present in the endoplasmic reticulum, but that SPH occurs at substantial levels in Golgi (12%) and particularly in plasma membrane (19%, ref. 52). Convincing evidence has emerged in recent years that SPH synthesis is localized in the plasma membrane (11,19-22,51), and our data are consistent with this observation.

In summary, it appears that SPH synthesis from ceramide and CDPcholine can be brought about in certain instances, particularly under in vitro conditions. On the cellular level, however, SPH synthesis is clearly independent of the cytosolic choline pool. Considering that the site of sphingomyelin synthesis is near the cell surface, use of a membrane-bound phosphocholine donor, such as phosphatidylcholine, may indeed be a necessity.

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METHODS

Rapid Analysis of Jojoba Wax Fatty Acids and Alcohols After Derivatization Using Grignard Reagents

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This method, based on the action of Grignard reagents, is an original approach to wax derivatization and differs from the methods previously described, which were essentially based on wax alcoholysis. Grignard reagents, especially magnesium ethyl bromide, react on ester functions to turn the wax constituents into primary and tertiary alcohols, the latter being the fatty acid derivatives. The mixture of these alcohols is analyzed by a single gas chromatographic injection. The overall time, about 1.5 hr, makes this method suitable for routine analysis. It could be also considered for analyzing low carbon condensation organic acid esters.
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The development of jojoba farming *(Simmondsia chinensis)* throughout the world and the many applications of its wax led us to seek rapid methods of analyzing its principal constituents that would be suitable for a large number of samples. First, we developed a method involving the rapid derivatization of fatty acids and alcohols into ethyl esters and acetates, respectively, followed by a single gas chromatographic (GC) analysis (1). A simplified method, which is a variation of the one above, has recently been proposed, i.e., reduction in the time taken for fatty acid ethanolysis, elimination of the fatty alcohol esterification step, injection of the fatty acid ethyl esters and free fatty alcohols mixture (2). These two methods avoid the major inconveniences of methods previously proposed by Miwa (3-6) and others (7,8), namely, prohibitive length of derivatization procedure and/or the need for two GC analyses.

This work, which further reduces analysis time, involves an original derivatization method using the reaction of organo-magnesium compounds on ester functions, to turn the constituents of the wax into primary and tertiary alcohols, the latter being the fatty acid derivatives. The mixture containing these alcohols undergoes a single GC analysis, as before.

MATERIALS AND METHODS

Reagents. Anhydrous ethanol, benzene and diethyl ether were analytical grade solvents (Prolabo, Paris, France). The ethyl ether was dried over $CaCl_2$, then distilled over sodium, stored on $CaCl_2$ and filtered before use. The organo-magnesium compounds used (Aldrich Chimie, Strasbourg, France) are supplied in 3 M solution in ethyl ether. The jojoba wax was supplied by the CEA (Marcoule, France); its acidity was 0.3%.

Thin layer chromatography (TLC). These analytical or preparative techniques have been described previously (1). In the present method, the solvent is a hexane/ethyl ether mixture (90:10, v/v), and the Rf of the wax is then 0.65.

Derivatization with magnesium ethyl bromide. Each sample is treated in three perfectly dry 30-ml test tubes. Wax derivatization is carried out in tube no. 1 with a ground neck fitted with a $CaCl_2$ trap.

Four drops of jojoba wax are introduced from a Pasteur pipette, along with 4 ml of anhydrous ethyl ether and 1.5 ml of the 3 M ethereal magnesium ethyl bromide solution. The mixture is placed on a vibrating stirrer for 10 min at room temperature; the excess reagent and the magnesium complexes are destroyed by adding 1 ml of N aqueous HCl and shaking for 1 min; 10 ml of distilled water is added and stirring is continued for a further 10 min; the upper ethereal phase is transferred to tube no. 2 containing 10 ml of a 2% aqueous $NaHCO_3$ solution and stirred for 10 sec on a vortex; the upper ethereal phase is filtered over anhydrous sodium sulfate into tube no. 3; the ethereal phase is evaporated till dry in a stream of nitrogen and the ethyl ether is replaced by 4 ml of hexane; one μl of the hexane solution containing the tertiary and primary alcohols is analyzed by GC.

Reference derivatization. This method involves two steps: ethanolysis of the wax (derivatization of fatty acids into ethyl esters) and extraction of the ethyl esters and free fatty alcohols. Each sample is treated in two 30-ml test tubes.

Ethanolysis takes place in tube no. 1 equipped with an air condenser fitted with a $CaCl_2$ trap. Four drops of jojoba wax are introduced from a Pasteur pipette, along with 0.1 ml of benzene and 1 ml of 5 N ethanolic HCl solution obtained by bubbling gaseous HCl through anhydrous ethanol; the mixture is refluxed for 25 min in an oil bath at 95 C.

The derivatives are extracted as follows: the solvents and HCl are evaporated in a stream of nitrogen for 10 min at room temperature; 6 ml of demineralized water and 4 ml of hexane are added, and this is stirred on a vortex for 10 sec; the supernatant is transferred by syringe into tube no. 2 containing anhydrous sodium sulfate. One μl of the hexane solution containing the mixture of fatty acid ethyl esters and free fatty alcohols is injected directly into the chromatograph.

GC analysis of wax derivatives. The solutions containing the wax derivatives (either ethyl esters and free fatty alcohols in the reference method or primary and tertiary fatty alcohols where derivatization uses organo-magnesium compounds) are analyzed on a Carlo Erba 4160 chromatograph equipped with a flame ionization detector, connected to a Delsi Enica 10 integrator. The constituents

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are separated on a DB Wax 30 W (J-W) fused silica capillary column with the following characteristics: length 30 m; internal diameter 0.317 mm; phase, Carbowax 20 M; film thickness 0.25 μm; injector-splitter at 250 C, detector at 275 C and oven 200 C except for mixture containing aromatic tertiary alcohols (220 C); carrier gas, helium; flow rate, 3 ml/min; splitting ratio 1/50.

RESULTS AND DISCUSSION

Derivatization reaction. The reactivity of Grignard reagents with respect to ester functions and their application in organic synthesis has been known for a long time; however, their use in organic analysis is very rare. Brockerhoff (9) astutely used magnesium methyl bromide to study the stereospecific distribution of triglyceride fatty acids in oils. This method consists in partly breaking down the triglycerides into partial glycerides, with the fatty acids being converted into tertiary fatty alcohols as shown on Scheme 1.

According to the reaction in Scheme 1, two organomagnesium molecules are required to transform one wax ester molecule. The reaction takes place in an anhydrous medium, hence particular care must be taken when drying the solvent. The complexes formed during the first step, along with the excess reagent, are instantaneously hydrolyzed in an acidic medium during the second step. The primary and tertiary alcohol series obtained constitute the wax derivatives. In practice, there must be a large amount of excess reagent in the reaction medium to ensure a complete reaction and trap any traces of water, which destroy this reagent; we established conditions leading to a quantitative reaction. In the case of ethyl magnesium bromide, when starting from a jojoba wax sample in the range of 1 g, we noted that the recovery of the products after the extraction step is quantitative. Moreover, the analysis of the extracted compounds by quantitative TLC (1) shows that the amount of products located in the three spots detected on the plate (residual wax, primary and tertiary alcohols) corresponds to the weight really deposited, using deposits of known amounts of standard (jojoba wax and pure derived alcohols).

Choice of the organo-magnesium reagent. It should be noted regarding the tertiary fatty alcohols that the nature of R″, the organo-magnesium compound radical, directly affects their molecular weights and, consequently, their chromatographic retention times. In effect, the carbon condensation of a derived tertiary alcohol is increased by twice the condensation of R″, compared with that of the corresponding fatty acid. Given this, particularly, the choice of R″ must be guided by the carbon condensation, but also the reactivity of the organo-magnesium compound and, possibly, by its commercial price and availability, although preparation of the reagent in the laboratory is no problem. Of the reagents tested, magnesium ethyl bromide was chosen because it gives the best results (no side products, fast reaction). With n-butyl and benzyl magnesium bromides, the reaction times are increased too much; by contrast, the reaction of methyl magnesium bromide is very fast, but gives side products.

Optimization of the reaction time was undertaken at room temperature with 1.5 equivalents of ethyl magnesium bromide. The time course study of reaction shows that wax conversion is complete after 10 min (Table 1).

GC analysis of derivatives. The GC analysis of jojoba wax derivatives obtained with magnesium ethyl bromide was carried out directly with a single injection on a commercially available fused silica capillary column (see Materials and Methods).

First, the study required the preparation of the 2 series of fatty alcohols by preparative TLC. The fact that the Rf values of the primary and tertiary alcohols are clearly distinct (0.10 and 0.40, respectively) makes it possible to apply large quantities while avoiding the reciprocal

TABLE 1

Time Course Study of Derivatization Reaction for Several Grignard Reagents (R″MgBr): Wt% of Residual Wax Determined by Quantitative TLC

	Reaction time (min)				
R″	5	10	30	60	120
Methyl	tr[a]				
Ethyl	5	tr[a]			
n-Butyl		60	20	5	1
Benzyl		35	10	2	tr[a]

R″: hydrocarbon radical of Grignard reagent.
[a]tr: <1% residual wax.

$$R{-}\underset{\underset{O}{\|}}{C}{-}OCH_2R' + 2\,R''MgBr \xrightarrow{1)\ Et_2O} R{-}\underset{R''}{\overset{R''}{C}}{-}O{-}MgBr + R'CH_2O{-}MgBr \xrightarrow{2)\ H_2O/HCl} R{-}\underset{R''}{\overset{R''}{C}}{-}OH + R'CH_2OH$$

wax esters — Grignard reagent — intermediate complexes — tertiary alcohols — primary alcohols

where R = fatty acid chain, $R'CH_2$ = fatty alcohol chain and R″ = hydrocarbon radical of Grignard reagent.

SCHEME 1

contamination of fractions. Hence, the separation of the two families, followed by GC analysis, presents no problem whatsoever. Identification of the GC peaks obtained is simple; regarding the series of primary alcohols, reference should be made to the previous work (2) in which the wax was derivatized to yield fatty acid ethyl esters and free fatty alcohols. Chromatographic analysis of the primary alcohols is not, therefore, modified by the choice of organo-magnesium compound, although this is not the case, of course, with tertiary alcohols.

The chromatogram of the diethyl alkyl carbinols (tertiary alcohols) is similar to that of the ethyl esters derivatized from the same fatty acids, to within the retention times. Identification was completed by referring to the chromatograms of diethyl alkyl carbinols obtained through similar treatment with a mixture of known triglycerides (the standard mixture composed of coconut, palm, rapeseed and peanut oils contains the main fatty acids of jojoba wax). In this respect, it should be noted that derivatization using magnesium ethyl bromide could constitute a new analysis method for determining the fatty acid composition of oils.

A comparative study of the separate chromatograms for the two categories of alcohols with that obtained for

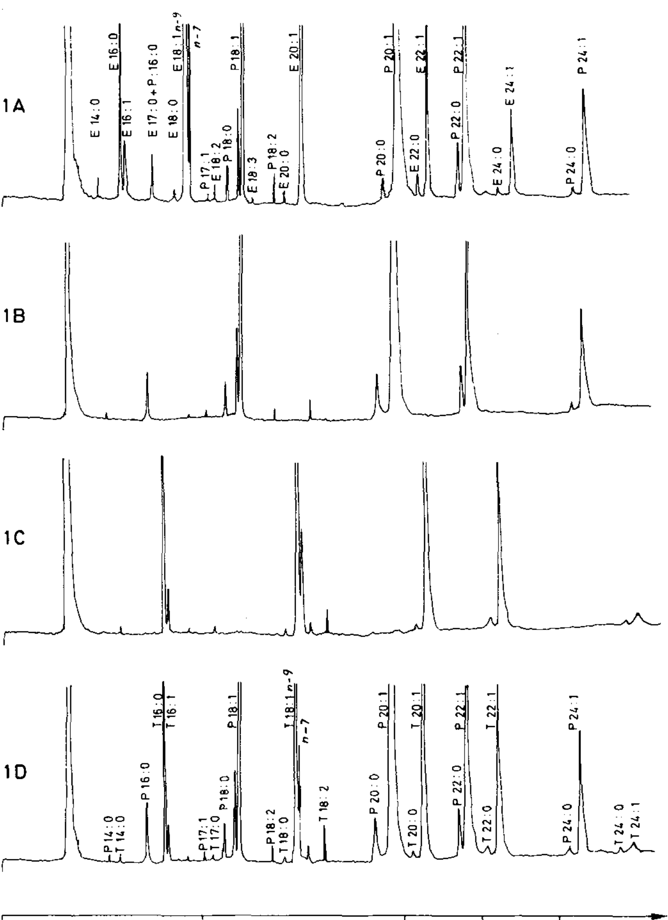

FIG. 1. Gas chromatograms of derived jojoba wax samples (DB Wax 30W). 1A, reference procedure (fatty acid ethyl esters + primary fatty alcohols); 1B, primary fatty alcohols; 1C, tertiary fatty alcohols; 1D, primary and tertiary fatty alcohols. Chart: 5 mm/min from 0 + 1000 sec and 1 mm/min after 1000 sec. Labeled peaks: E, fatty acid ethyl esters; P, primary fatty alcohols; T, tertiary fatty alcohols.

derivatized jojoba wax reveals that peak resolution is highly satisfactory (Fig. 1). For fatty acid derivatives, it should be noted that ethyl ester retention times are lower than those for the corresponding diethyl alkyl carbinols; they are delayed compared to primary alcohols, unlike in the reference method (Fig. 1). In effect, the difference in carbon condensation between a primary alcohol and the corresponding tertiary alcohol is equal to 2 n, where n is the number of carbon atoms of the magnesium alkyl or aryl compound. In the case of magnesium ethyl bromide, the difference equal to 4 carbon atoms explains why the chromatographic retention of the diethyl alkyl carbinol is greater than that of the corresponding primary fatty alcohol and, all the more so, than that of the ethyl ester.

It would have been more satisfactory to have obtained chromatograms in which the series of primary alcohols was completely eluted before that of the tertiary alcohols. The n-butyl and benzyl magnesium bromides were tested in light of this aim. The n-butyl radical, which adds 8 carbon atoms, brings to 24 the carbon condensation of the shortest tertiary alcohol derivatized from the C16 acid. Therefore, 24 is the carbon condensation of the heaviest primary alcohol. Although the branched structure lowers retention time compared to a linear homologue, we were unable to achieve this objective. In addition, the time taken for analysis would have been considerably longer. In aliphatic series, the R″ > n-butyl homologues were not tested because of the low reactivity of the corresponding bromo magnesium compounds.

The reactivity of the magnesium benzyl bromide is comparable to that of the n-butyl reagent (Table 1), but it adds 14 carbon atoms to the derivatized fatty acids; further, elution of this series of tertiary alcohols requires that the time taken for GC analysis be doubled, despite the high temperature, which is almost the limit for the stationary phase (oven temperature of 220 C, instead of the usual 200 C). In spite of the prohibitive length of the analysis time (more than 2 hr), the two groups of wax derivatives are still not totally separated. Consequently, derivatization using magnesium ethyl bromide is the quickest and most effective method from both a reactivity and chromatographic analysis point of view. Even if analysis time is slightly increased compared to the reference method (fatty acid ethyl esters), this method offers incomparable simplicity.

Jojoba wax composition was calculated from the average of five derivatizations and two GC analyses for each derivatization (Table 2). Derivatization of the wax constituents was also carried out according to the reference method (2). The compositions found from each of the methods are identical within the limits of unavoidable experimental error.

To conclude, the method proposed, based on the action of Grignard reagents, is an original approach to the derivatization of waxes and differs from the methods previously described, which were essentially based on wax alcoholysis. It also offers several advantages compared to the method we recently put forward: use of relatively cheap commercially available reagents, a single derivatization step and almost immediate reaction; operations carried out at room temperature, not even requiring a thermostatically controlled bath; very simple glassware adapted to the analysis of numerous samples. Complete sample analysis can be done in about 1.5 hr, enabling an

TABLE 2

Composition of Primary and Tertiary Fatty Alcohols Derived from Jojoba Wax

	Reference method (2)[a]	Derivatization by Et Mg Br[b]
Acids		
16:0	1.8	1.5
16:1	0.3	0.3
17:0	—	0.1
18:0	0.1	0.1
18:1[c]	13.9	13.0
18:2	0.4	0.5
18:3	0.1	0.1
20:0	0.1	0.2
20:1	70.4	70.8
22:0	0.2	0.2
22:1	11.3	11.8
24:0	0.1	0.1
24:1	0.9	1.0
Others	0.4	0.3
Alcohols		
16:0	0.2	0.2
17:1	tr[d]	0.1
18:0	0.2	0.2
18:1	1.1	1.0
18:2	tr	tr
20:0	0.3	0.3
20:1	51.1	51.2
22:0	1.0	1.1
22:1	39.1	38.5
24:0	0.2	0.2
24:1	6.1	6.2
Others	0.7	1.0

[a]Wt% of fatty acid ethyl esters and primary fatty alcohols determined by gas chromatography, DB wax 30 W.

[b]Wt% of primary fatty alcohols and tertiary alcohols derived from fatty acids. Mean of 5 replicate runs (2 gas chromatographic analyses for each).

[c]Sum of isomers n-9 and n-7.

[d]tr: trace amounts <0.05%.

organized operator to treat several samples per day. Furthermore, this method could be considered for analyzing low carbon condensation organic acid esters, since the choice of a suitable magnesium compound would make it possible to obtain a heavier tertiary alcohol, thus avoiding losses; for those absorbing in UV, high pressure liquid chromatographic analysis should be easy.

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High Performance Liquid Chromatographic Analysis of 1-Alkyl-2-acyl- and 1-Alkyl-3-acyl-*sn*-glycerols[1]

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A high performance liquid chromatographic (HPLC) method is described for separation and quantitation of 1-alkyl-3-acyl- and 1-alkyl-2-acyl-*sn*-glycerol, products of the detritylation reaction of 1-alkyl-2-acyl-3-trityl-*sn*-glycerol. The alkyl glycerides were separated on a 25 cm × 4.6 mm ID column packed with ∼5-6 μm silica and eluted isocratically with isooctane/isopropanol (98:2, v/v) as mobile phase. The good separation and linear refractive index (RI) detector responses using cholesterol as an internal standard indicated the applicability of the method not only for the quantitative determination of the alkylglycerols but also for their semipreparative isolation. This HPLC method shows excellent reproducibility and accuracy and is applicable to other types of glycerides such as mono- and diacylglycerols.
Lipids 22, 362-365 (1987).

Common precursors for the stereospecific synthesis of 1-alkyl- neutral and phospholipids are 1-alkyl-2-acyl-3-trityl-*sn*-glycerols in which the trityl group is used to protect the hydroxyl group at the 3-position of glycerol (1,2). The next step in the synthetic sequence is removal of the trityl protecting group, under general acid catalysis, to yield 1-alkyl-2-acyl-*sn*-glycerols as key intermediates (Scheme 1). Acylation or phosphorylation of the latter glycerides gives the desired 1-alkyl- neutral or phosphoglyceride, respectively. Quite often, however, removal of the trityl protecting group is complicated by the concomitant migration of the 2-acyl moiety to yield the isomeric 1-alkyl-3-acyl-*sn*-glycerol derivative (3-6). As a consequence of this acyl rearrangement, it is necessary to separate and quantify these isomeric alkyl glycerides prior to further modification. A number of analytical methods, including column chromatography (7), thin layer chromatography (TLC) (8,9), high performance liquid chromatography (HPLC) (10,11) and gas liquid chromatography (GLC) (12) have been reported for separation and quantitative determination of mixtures of mono-, di- and triacylglycerols. For the isolation of glycerides for subsequent chemical manipulation, however, HPLC methods are more advantageous than other analytical methods because of their nondestructive nature, greater range of sample capacity and ease or rapidity of operation. Moreover, HPLC has the potential not only for isolating partial glycerides from reaction mixtures but also for their subsequent purification. This paper describes the separation and quantitative determination of mixtures of various 1-alkyl-2-acyl- and 1-alkyl-3-acyl-*sn*-glycerols as well as their semipreparative isolation by normal phase HPLC with isocratic elution.

HO–[O-ALK / OH] —1) TrCl, 2) RCOCl→ ACYL-O–[O-ALK / O-Tr] ——→ ACYL-O–[O-ALK / OH] + HO–[O-ALK / O-ACYL]

1,2-Isomer 1,3-Isomer

SCHEME 1

MATERIALS AND METHODS

Materials. 1-Alkyl-2-acyl-3-trityl-*sn*-glycerols were synthesized in our laboratory starting with D-mannitol (99%) obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Oleic acid (>99.6%) used for the synthesis of oleoyl chloride (12) for acylation of the secondary hydroxyl group of glycerol was from Nippon Oil and Fats Co. (Amagasaki, Japan). Acetyl chloride (98.5%) was obtained from J.T. Baker Chemical Co. (Philipsburg, New Jersey); palmitoyl chloride (98%) and benzoyl chloride (99%) were obtained from Aldrich. Stearoyl chloride was synthesized in our laboratory. Triphenylmethanol used for the synthesis of triphenylchloromethane for tritylation of the primary hydroxyl group of glycerol was obtained from Eastman Organic Chemicals (Rochester, New York). The detritylation catalyst, boron trifluoride etherate, was obtained from Eastman Kodak Co. (Rochester, New York) and distilled prior to use. Isooctane and isopropanol used for HPLC separations were obtained from American Burdick & Jackson (Muskegon, Michigan). Cholesterol standard (>98%) was obtained from NuChek Prep (Elysian, Minnesota).

Analytical system. (a) Chromatograph: The solvent delivery system consisted of a Beckman Model 110A solvent delivery module equipped with a Waters Differential Refractometer Model R401 detector (Waters Associates, Milford, Massachusetts) and an Altex 210 injector. (b) Analytical HPLC column: Zorbax SIL, 4.6 mm ID × 25 cm (∼5-6 μm, DuPont Co., Wilmington, Delaware). Sepralyte Diol, 4.6 mm ID × 25 cm (5 μm, Analytichem International, Harbor City, California). Semipreparative HPLC column: Dynamax Prepacked Silica Column, 10 mm ID × 25 cm (8 μm, Rainin Instrument Co., Woburn, Massachusetts). (c) Integrator and recorder: Chromatopac C-R3A (Shimadzu Co., Columbia, Maryland). (d) HPLC conditions: The samples were eluted isocratically with 98% isooctane/2% isopropanol (v/v) at a flow rate of 1 ml/min (analytical HPLC) or 3 ml/min (semipreparative HPLC). Injection volumes were 20 μl (analytical HPLC) or 100 μl (preparative HPLC), and the samples were injected via loop injectors.

Syntheses. 1,2-Isopropylidene-*sn*-glycerol was prepared from D-mannitol via the lead tetraacetate cleavage of 1,2,5,6-diisopropylidene-D-mannitol to 1,2-isopropylidene-*sn*-glyceraldehdyde and subsequent reduction of the aldehyde with sodium borohydride according to the procedure established by Eibl (14). $[\alpha]_D^{25}$ + 14.5° (0.169 mg/ml, CH_3OH). Infrared spectrum: 3425 cm^{-1} (OH), 1378 and 1368 cm^{-1} (isopropyl).

[1]Presented in part at the AOCS annual meeting, Honolulu, Hawaii, May 1986.

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3-Hexadecyl-*sn*-glycerol was prepared from 1,2-isopropylidene-*sn*-glycerol by reaction with sodium hydride in dimethylformamide followed by alkylation with hexadecylbromide, mp 64.9–67.0 C, lit. mp 65–67 C (13). Infrared spectrum 3405, 3326 and 3240 cm^{-1} (OH), 1127 cm^{-1} (C-O-C).

3-Hexadecyl-*sn*-glycerol was converted to its enantiomer, 1-hexadecyl-*sn*-glycerol, according to the method of Chacko and Hanahan (15). Reaction of 3-hexadecyl-*sn*-glycerol with p-toluene-sulfonyl chloride yielded the ditosylate derivative, which underwent SN_2 displacement with acetate anion to give 1-hexadecyl-2,3-diacetoxy-*sn*-glycerol. Alkaline hydrolysis of the diacetate gave the desired 1-hexadecyl-*sn*-glycerol, mp 64.4–66.0 C, lit. mp 65–67 C (15). Infrared spectrum: 3405, 3333 and 3240 cm^{-1} (OH), 1130 cm^{-1} (C-O-C).

1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol was synthesized by tritylation at the 3-position of 1-hexadecyl-*sn*-glycerol followed by acylation at the 2-position of the resultant 1-hexadecyl-3-trityl-*sn*-glycerol (16). Infrared spectrum: 3085, 3055 and 3020 cm^{-1} (triphenylmethyl), 3000 cm^{-1} (-CH=CH-), 1733 cm^{-1} (-C=O). Other 1-hexadecyl-2-acyl-3-trityl-*sn*-glycerols were prepared in a similar manner.

Detritylation reaction. The detritylation reaction was carried out using a modification of Hermetter and Pattauf's method (16). 1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol (40 μmol), BF_3-CH_3OH catalyst (40 μmol) and methylene chloride (2 ml) were placed into a 4-ml glass vial equipped with a magnetic stirrer. After the vial was sealed with a viton seal under nitrogen, the mixture was stirred for ~30–60 min at 22 C. Similar reactions were performed with other 1-hexadecyl-2-acyl-3-trityl-*sn*-glycerols having various acyl groups at the 2-position (Table 2). The corresponding detritylation products, 1-hexadecyl-2-acyl-*sn*-glycerol and 1-hexadecyl-3-acyl-*sn*-glycerol were isolated by semipreparative HPLC described above.

Internal standard calibration. Standards containing 10 to 40 μmol of 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, 1-hexadecyl-2-oleoyl-*sn*-glycerol, 1-hexadecyl-3-oleoyl-*sn*-glycerol and cholesterol (internal standard) were prepared in 1 ml of isooctane/isopropanol (98:2, v/v). The diradyl glycerol solution and standard cholesterol solutions were mixed (~50–200 μl) and analyzed by analytical HPLC. The weight ratios (w/w) of the alkyl glycerol (W) to the internal standard (WS) were varied between 0.1 and 1.4. Similar analyses were performed for the other diradyl glycerols having different acyl groups at the 2-position (Table 2).

RESULTS AND DISCUSSION

1-Alkyl-2-acyl glycerols are one of the more important classes of intermediates for the syntheses of 1-alkyl phospholipids. Much effort has been given to the synthesis and characterization of this class of lipid since migration of the 2-acyl unit to more thermodynamically stable 1,3-isomer is very facile. It is well known that the isomerization of the 1,2-isomer to 1,3-isomer is subject to general acid or base catalysis (17). As a result of this rearrangement, rapid methods are needed for analysis and purification of these partial glycerides. In this study, we have established an excellent separation method for this class of alkyl glycerol by normal phase HPLC.

HPLC and internal standard. HPLC separations of a test mixture consisting of (a) 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, (b) triphenylmethyl alcohol, (c) 1-hexadecyl-3-oleoyl-*sn*-glycerol, (d) 1-hexadecyl-2-oleoyl-*sn*-glycerol and (e) cholesterol (IS) are shown in Figure 1. As the chromatograms indicate, some differences can be seen in the separations between the silica column and the diol column used in this study. The difference in retention times between the 1,3- and 1,2-isomers found in the HPLC on silica was larger than on the diol column, and no peaks for detritylation reaction products were found after elution of the 1,2-isomer. The data suggested the possibility of using a silica HPLC separation for the quantitative analysis of detritylation reaction mixtures and for preparative HPLC. Cholesterol is a suitable internal standard for quantitation.

Figure 2 shows the effect of solvent strength on the separation of a test mixture. As the results indicate, a better resolution of the five components in the test mixture was obtained with an increase in the amount of isooctane in the solvent system. However, it should be noted that the decreased solubility of triphenylmethanol with this mobile phase composition caused deterioration of the HPLC column and interfered with the HPLC analysis. The efficiency of the column was restored after washing it with 90:10 isooctane/isopropanol. Accordingly, it seems that a solvent system of isooctane/isopropanol of 98:2 composition is the preferred solvent system.

Figure 3 gives the RI detector responses of (a) 1-hexadecyl-2-oleoyl-*sn*-glycerol, (b) 1-hexadecyl-3-oleoyl-*sn*-glycerol and (c) 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol. The

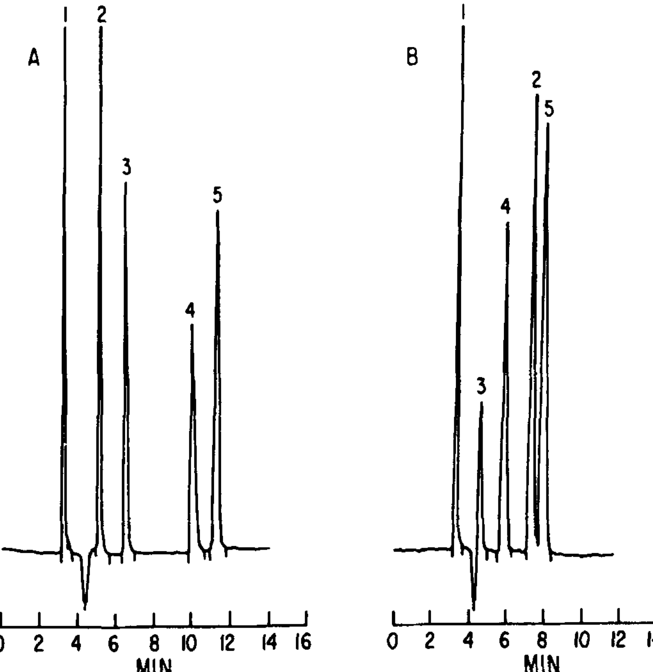

FIG. 1. HPLC chromatograms of a test mixture. Panel A, silica column; panel B, diol column. Compounds separated: 1) 1-hexadecyl-2-oleyl-3-trityl-*sn*-glycerol, 2) triphenylmethanol, 3) 1-hexadecyl-3-oleoyl-*sn*-glycerol, 4) 1-hexadecyl-2-oleoyl-*sn*-glycerol and 5) cholesterol.

data are plotted as weight ratio of the alkylglycerol to the internal standard (W/W_{IS}) along the ordinate and HPLC area ratios (A/A_{IS}) along the abscissa. In the case of the RI detector, good linear relation and sensitivity were obtained in the weight ratio range of 0 to 1.2 (alkylglycerol/standard). Detector response factors were calculated from the slope of each line, and the standard deviations obtained for the four different test mixtures (Table 1) (A, 1.7%; B, 1.3%; C, 1.4%, respectively) indicate that the method has good reproducibility and accuracy for the quantitative analysis of alkylglycerols.

In the quantitative analysis of the detritylation reaction, the mole fraction of each material was calculated from the following equation, where each weight ratio was obtained from each HPLC area ratio using the response factors given in Figure 3.

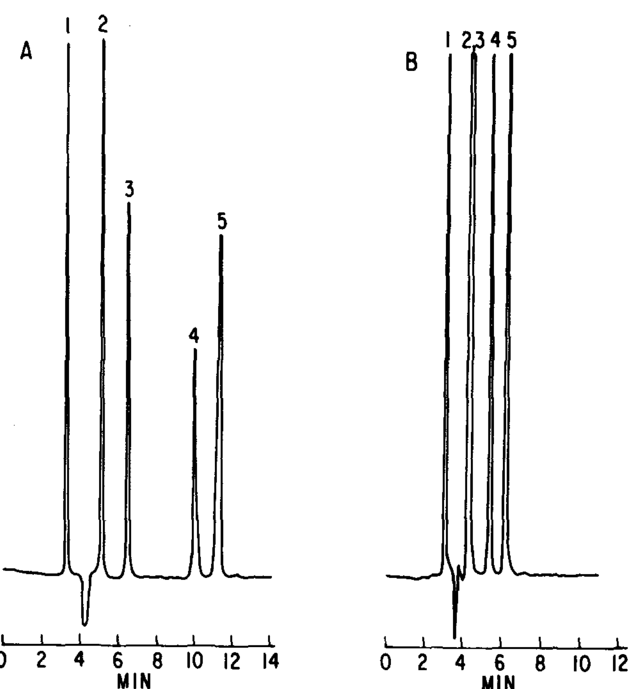

FIG. 2. Effect of solvent on the separation of a test mixture. A) Isooctane/isopropanol, 98:2 (v/v); B) isooctane/isopropanol, 95:5 (v/v). 1) 1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, 2) triphenylmethanol, 3) 1-hexadecyl-3-oleoyl-*sn*-glycerol, (4) 1-hexadecyl-2-oleoyl-*sn*-glycerol and 5) cholesterol.

$$F_A = \frac{\frac{WA/WIS}{MA}}{\frac{WA/WIS}{MA} + \frac{WB/WIS}{MB} + \frac{WC/WIS}{MC}}$$

where F is the mole fraction of the alkylglycerol in the reaction mixture. WA/WIS, WB/WIS and WC/WIS are the weight ratios of materials A, B and C, respectively. MA, MB and MC are the molecular weights of materials A, B and C, respectively.

Retention time of alkylglycerols. The retention times obtained using the conditions given in Materials and Methods for 1-hexadecyl-2-acyl-3-trityl-, 1-hexadecyl-3- and 1-hexadecyl-2-acyl-*sn*-glycerols containing various fatty acyl chains are given in Table 2. As the data show, the retention times of the alkylglycerols increased with a decrease of the carbon chain length of the fatty acyl residue due to an increase of its polarity. Separation of mixtures of various molecular species for each lipid class

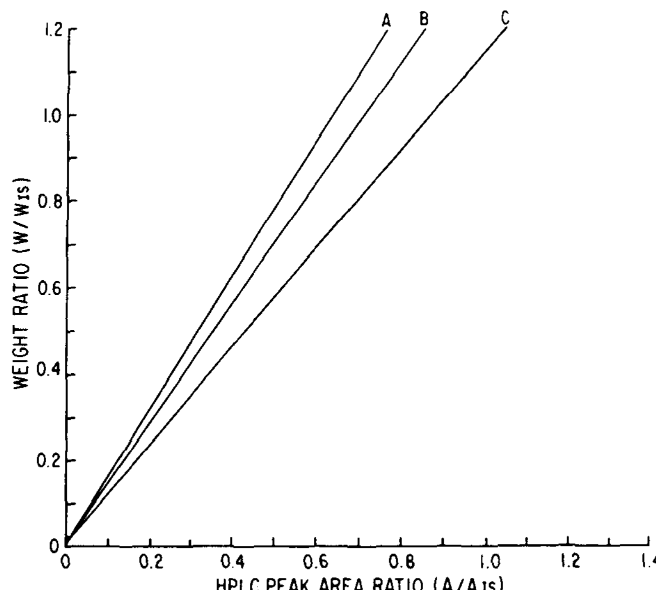

FIG. 3. Internal standard calibrations for A) 1-hexadecyl-2-oleoyl-*sn*-glycerol, B) 1-hexadecyl-3-oleoyl-*sn*-glycerol and C) 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol.

TABLE 1

Quantitative Analyses of Alkylacylglycerols

Standard mixture	A: 1,2-(MF)[a]			B: 1,3-(MF)			C: 1,2,3-(MF)		
	Found[b]	Actual[c]	Ratio	Found	Actual	Ratio	Found	Actual	Ratio
Sample A	0.394	0.387	1.018	0.378	0.381	0.992	0.229	0.232	0.987
Sample B	0.445	0.438	1.016	0.428	0.430	0.995	0.126	0.131	0.962
Sample C	0.483	0.479	1.008	0.232	0.235	0.987	0.286	0.286	1.000
Sample D	0.234	0.240	0.975	0.482	0.472	1.021	0.285	0.288	0.990
Mean ± S.D.									
± 0.014		1.004 ± 0.017			0.999 ± 0.013			0.985	

[a]MF, mole fraction. Average of three observations. A, 1-Hexadecyl-2-oleoyl-*sn*-glycerol; B, 1-hexadecyl-3-oleoyl-*sn*-glycerol; C, 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol.

[b]Mole fraction determined by semipreparative HPLC isolation.

[c]Mole fraction determined by analytical HPLC using response factors given in Fig. 3.

TABLE 2

HPLC Retention Times of Glycerides Containing Various Fatty Acids

Fatty acid at 2-position	Retention time (min)[a]		
	1,2,3[b]	1,3[c]	1,2[d]
Acetic	3.94	18.04	27.84
Benzoic	3.58	9.38	14.22
Palmitic	3.19	6.85	10.92
Stearic	3.16	6.50	10.16
Oleic	3.17	6.56	10.21
Oleic[e]	3.37	9.08	13.28

[a]Average of three observations—analytical HPLC silica column: 98:2 (v/v) isooctane/isopropanol, flow rate of 1 ml/min.
[b]1-Hexadecyl-2-acyl-3-trityl-*sn*-glycerol.
[c]1-Hexadecyl-3-acyl-*sn*-glycerol.
[d]1-Hexadecyl-2-acyl-*sn*-glycerol.
[e]1,2,3-, triolein; 1,3- and 1,2-, diolein.

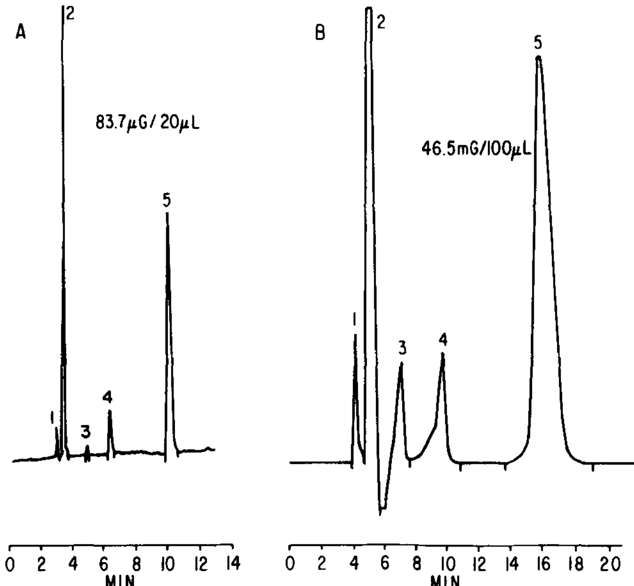

FIG. 4. HPLC chromatograms of detritylation reaction product. A) Analytical HPLC silica column—98.2 (v/v) isooctane/isopropanol, flow of 1.0 ml/min; B) semipreparative HPLC silica column—98:2 (v/v) isooctane/isopropanol, flow of 3 ml/min. 1) 1-Hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol, 2) methyl triphenylmethyl ether, 3) triphenylmenthanol, 4) 1-hexadecyl-3-oleoyl-*sn*-glycerol and 5) 1-hexadecyl-2-oleoyl-*sn*-glycerol.

was not always possible with the HPLC conditions used. However, a complete separation of individual isomeric lipid species, 1,2,3-, 1,3- and 1,2-glycerols, was obtained under the conditions. The separation among these three derivatives increased with a decrease in the carbon chain of fatty acid acyl residue, with the largest separations obtained with the acetyl derivatives. The last entry in Table 2 shows that the method is also applicable to the separation of mono-, di- and triacylglycerols, giving the same order of elution as observed with the alkylacylglycerols. However, substitution of an acyl residue for alkyl increases retention times for all three acyl glycerides. This indicates the decreased polarity of an alkoxy group compared to an acyloxy substituent.

Preparative HPLC application. To obtain information on the utility of preparative column HPLC for the isolation of the 1,2- and 1,3-alkylacyl isomers, both the analytical and preparative HPLC columns were compared at different sample loadings (Fig. 4). The sample used for these chromatograms was obtained by the detritylation reaction of 1-hexadecyl-2-oleoyl-3-trityl-*sn*-glycerol at a reaction temperature of 22 C, in methylene chloride solvent, BF_3-CH_3OH to glyceride molar ratio of 1:1 and a reaction time of 5 min. As the data show, a comparable separation and sensitivity was obtained on the semipreparative silica column as with the analytical silica column. This result suggested the further possibility of using HPLC for the semipreparative isolation and purification of the isomeric 1-alkyl-2-acyl and 1-alkyl-3-acyl-*sn*-glycerols listed in Table 2.

At the present stage of the study, only the semipreparative column was used for this purpose. In the future, we are planning to use preparative columns with larger ID for isolation of larger amounts of synthetic mixed alkylacylglycerol isomers.

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Quantitation of Baboon Lipoproteins by High Performance Gel Exclusion Chromatography

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High performance liquid chromatography with gel exclusion columns was used for quantitative measurement of plasma lipoproteins. A combination of columns TKS 4000 PW and 3000 PW gave good separation of very low (VLDL), low (LDL) and high (HDL) density lipoproteins. The area under each lipoprotein peak detected by absorbance at 280 nm was measured by digitizing and was expressed as cm^2. Purified lipoprotein standards isolated by ultracentrifugation were also chromatographed in increasing concentrations. The area under the lipoprotein standard peak was linearly related to the amount of total protein over a wide range. The areas of most of the measured plasma lipoproteins were within the linear range. The relationship between the area and the amount of protein for each standard was used to quantitate the amount of protein and was expressed as mg/dl plasma. This technique is simple and requires a small amount of plasma. The validated technique was applied to a large population of pedigreed baboons. An average plasma lipoprotein profile of feral baboons on the chow diet was characterized by a high level of HDL (90.9 ± 30.7 mg/dl) with a lesser amount of LDL (29.1 ± 13.2 mg/dl). VLDL was present in much lower concentration (8.6 ± 2.6 mg/dl). Feeding a high cholesterol and high saturated fat (HCHF) diet raised both LDL (1.5-fold) and HDL levels (1.3-fold) without changing VLDL levels. Progeny of sires with low response to dietary cholesterol increased their HDL protein when challenged with HCHF diet without any change in their LDL or VLDL. Progeny of high-responding sires, however, had increases in both their HDL and LDL levels when challenged with HCHF diet. The survey of lipoprotein profiles of the pedigreed baboon colony disclosed a number of animals with interesting and unusual lipoprotein patterns.
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Plasma concentrations of lipoproteins are affected by both genetic and environmental factors. Because plasma lipoproteins are involved in the pathogenesis of atherosclerosis, many efforts have been devoted to determining the factors that control their concentration and composition, both in humans and in animal models. Nonhuman primates have been widely used because of their close phylogenetic relationship to man. We have used the baboon (*Papio* sp.) because its lipid and lipoprotein metabolism is similar in many ways to that of humans, and because it exhibits a wide variety of plasma lipoprotein profiles (phenotypes) that are presumed to be of genetic origin. Since 1973, we have developed, by selective breeding and positive assortative mating, families of baboons with distinctive lipoprotein phenotypes. Genetic analyses have shown that some phenotypes are heritable (1) and are due to the effects of major genes (2). Most of these phenotypes are modulated by diet as well as by breeding.

The systematic study of both genetic and dietary effects on these lipoprotein phenotypes required a rapid quantitative method to measure concentrations of major lipoprotein classes in plasma. We used high performance gel exclusion liquid chromatography (HPLC), which has been shown to separate lipoproteins (3–5). A previous report described the qualitative validation of the method (6). This report describes quantitative validation of the method and its application to the measurement of lipoproteins in several families of pedigreed baboons with normal and abnormal lipoprotein phenotypes.

METHODS

Subjects. About 500 pedigreed baboons, the result of 10 years of selective breeding, were available for the survey of plasma lipoprotein phenotypes. For this analysis of results obtained by HPLC, we selected 96 feral animals and 97 progeny of 21 sires and 86 dams. Sires were selected on the basis of the response of their plasma cholesterol concentrations to an atherogenic diet rich in cholesterol and saturated fat. One group of sires (n = 7) had low responses, and a second group (n = 8) had high responses to the challenge diet. The third group (n = 6), called the control group, was selected randomly. The feral animals ranged from nine to 20 years in age at the time of sampling. Progeny were between three and five years old at the time of analysis. Blood was drawn while animals were consuming monkey chow, and again after consuming a diet enriched in cholesterol and saturated fat for seven weeks. The compositions of these diets are given in Table 1.

Isolation of plasma lipoproteins for HPLC. Animals fasted for 16 to 20 hr were immobilized with ketamine (10 mg/kg) and bled to obtain 3 ml of blood in tubes containing EDTA (1 mg/ml). The plasma was obtained by

TABLE 1

Compositions of Chow and High-Cholesterol, High-Fat (HCHF) Diets

Nutrients	Chow diet	HCHF diet[a]
Carbohydrates (% cal)	62	40
Protein (% cal)	28	20
Fat (% cal)	10	40
Energy (kcal per 100 g diet)	329	377
Cholesterol (mg/kcal)	0.03	1.7

[a]HCHF diet was prepared by mixing 81.4% (dry weight basis) of Purina monkey meal 5-5045-6 (a special mix with no added fat, dehydrated alfalfa, sodium chloride, ascorbic acid or retinyl acetate) with lard (16.5%), sodium chloride (1.1%), retinyl acetate (0.005%), ascorbic acetate (0.2%) and cholesterol (0.74%).

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centrifuging the blood in a low-speed centrifuge (TJ6B, Beckman Co., Palo Alto, California) at 3000 rpm (1900 × g) for 30 min at 6 C. Plasma (1.0 ml) was adjusted to a density of 1.21 g/ml by adding solid potassium bromide and was ultracentrifuged (Beckman L5-50 and L3-50) in a 50 Ti rotor at a speed of 45,000 rpm for 24 hr at 6 C. After ultracentrifugation, lipoproteins were harvested by pipetting the upper 3.0 ml. These lipoproteins were used for the characterization and quantitation of lipoprotein profiles by HPLC.

Separation of lipoproteins by HPLC. Plasma lipoproteins were separated by HPLC by using gel exclusion columns as reported previously (6). A Waters HPLC Model 204 with 6000A pump and UV detector (Model 440) (Waters Assoc., Milford, Massachusetts) was used. The lipoprotein peaks were detected by monitoring absorbance at 280 nm with a Perkin-Elmer Sigma 15 recorder-integrator (Perkin-Elmer Corp., Norwalk, Connecticut). Separation in Tris acetate buffer, 0.2 M, pH 7.0, was achieved by a combination of gel permeation columns, a guard column TSK GP WPC (100 × 7.5 mm), a TSK 4000 PW (600 × 7.5 mm) and a TSK 3000 PW (600 × 7.5 mm, Kratos, Westwood, New Jersey). A flow rate of 0.2 ml/min was used with a recorder speed of 0.1 cm/min. Each sample was injected in a volume of 300 μl (equal to 100 μl of plasma) by a Waters WISP maintained at 5 C in a refrigerated chromatography chamber (Powers, Hatboro, Pennsylvania). When fractions were collected, a Foxy fraction collector (ISCO, Lincoln, Nebraska) maintained at 5 C in a chromatography chamber was used.

Separation of lipoproteins by density gradient ultracentrifugation. Lipoproteins were separated by density gradient ultracentrifugation using a modified method of Redgrave et al. (7). Plasma samples (2.0 ml) were pipetted into polyallomer centrifuge tubes, and plasma was adjusted to d 1.30 g/ml by adding solid potassium bromide (0.430 g/ml). A discontinuous gradient was formed by layering 1.5 ml of d 1.21 g/ml KBr solution, followed by 1.5 ml of d 1.125 g/ml, 1.5 ml of d 1.070 g/ml, 2.0 ml of d 1.063 g/ml and 1.5 ml of d 1.019 g/ml KBr solutions. Finally, the contents were layered with 1.2 ml of saline solution (d 1.006 g/ml). The salt solutions were layered using a 22-gauge hypodermic needle attached to a syringe with the piston removed as described by Redgrave et al. (7). The samples were centrifuged for 24 hr at 39,000 rpm using an SW 41 Ti rotor at 6 C in a Beckman ultracentrifuge model L8-70 (Beckman).

After centrifugation, the tube was placed on a gradient tube fractionator (FS 101, Hoefer Scientific Instruments, San Francisco, California) and punctured in the side close to the bottom. Fluorinert FC-40 (Sigma Chemical Co., St. Louis, Missouri) was then pumped and the sample placed through an optical density monitor attached to a recorder (ISCO) using a peristaltic pump (Gibson Medical Electronics, Middleton, Michigan) and 0.4-ml fractions were collected. The fractions corresponding to different lipoprotein peaks, as monitored by absorbance, were pooled.

Isolation and chromatography of lipoprotein standards. Lipoprotein standards for identification and quantification of lipoprotein peaks separated by HPLC were separated by sequential ultracentrifugation as described previously (6). Very low density lipoproteins (VLDL), LDL and high density lipoprotein (HDL) were isolated at d 1.006 g/ml, d 1.019–1.063 g/ml and d 1.063–1.21 g/ml, respectively. An abnormal lipoprotein between LDL and HDL (HDL_1) was isolated from an animal positive for this lipoprotein at d 1.045–1.070 g/ml. Lipoproteins were floated at least two times at their respective densities by ultracentrifugation. The pure lipoprotein fractions used as standards gave single bands corresponding to their characteristic electrophoretic mobility on 1% agarose gel electrophoresis (8). Likewise, on HPLC, a single peak was obtained (6). The protein concentrations of lipoprotein standards were measured by the method of Lowry et al. (9). We then chromatographed standards by HPLC in varying concentrations and kept the conditions for HPLC similar to those used for plasma lipoprotein separation.

Quantitation of cholesterol in lipoproteins separated by density gradient ultracentrifugation and HPLC. To compare the recovery of cholesterol in lipoproteins separated by density gradient ultracentrifugation and HPLC, blood was obtained from seven animals (four on chow and three on HCHF diet) after an overnight fast. Plasma lipoproteins were fractionated by density gradient ultracentrifugation and HPLC as described earlier. Lipoproteins separated by both methods were assayed for cholesterol by an enzymatic method at a sensitivity of 1 μg cholesterol with a precision of ±4% using a cholesterol measurement kit (Sigma).

Quantitation of lipoprotein profiles. Lipoprotein profiles obtained after HPLC separation were digitized, and the area under each peak was expressed as cm^2. Initially the areas were measured by using a sonic digitizer (Science Accessories, Southport, Connecticut) attached to a minicomputer (Zobex, San Diego, California) with a video display unit (ADMS 3A+ Dumb terminal, Lear Siegler, Anaheim, California). The program was designed locally and integrated the area under the curve between any two arbitrary points plotted by digitizer. Later, the areas were measured by using the digital Paintbrush system (The Computer Colorworks, Sausalito, California) attached to an IBM PC computer (IBM, Boca Raton, Florida).

Standard lipoproteins separated by HPLC were digitized in a similar way, and the area under the peak was expressed as cm^2. This value of the peak was plotted against the Lowry protein values to determine the linear range of proteins in various lipoproteins. The relationship between the area and the amount of protein in each standard curve was used to quantitate the lipoprotein in corresponding peaks of the animal's lipoprotein profile separated by HPLC.

Statistical analysis. The data for densitometric areas and protein concentrations for each lipoprotein standard gave a straight line when fitted by the criterion of least squares. The correlation coefficients for these regression lines were highly significant ($P < 0.01$). These equations for straight lines were then used to calculate the lipoprotein concentrations in the plasma by using the densitometric areas. The data for lipoprotein cholesterol or protein measured by two methods were compared by using two-way analysis of variance (ANOVA) considering the two methods as two blocks and repeated measurements or animals as treatments. Similarly, the lipoprotein data for the chow and HCHF diet within each group were compared using two-way ANOVA considering lipoprotein protein concentration as two blocks and animals as treatments. Lipoprotein protein concentrations for groups were compared by one way ANOVA. After detection of

significant differences by ANOVA, individual means were compared using Duncan's New Multiple Range Test to see which pairs of means were different. The recovery of cholesterol in lipoproteins by two methods was compared using one-way ANOVA.

RESULTS

Identification of lipoproteins separated by HPLC. The lipoprotein peaks separated by HPLC were identified by comparing their retention times with those of purified lipoprotein standards and apoprotein composition as described previously (6). The lipoprotein patterns separated by HPLC and the cholesterol distribution among the fractions separated resembled those of density gradient ultracentrifugation (Fig. 1). As described previously (6), the first peak, which eluted at 82-90 min, corresponded to VLDL. It was followed by LDL with a retention time of 95-109 min and HDL with a retention time of 115-130 min. Albumin was eluted at 140 min; however, it was not present in any detectable amounts in these samples. In some cases a peak corresponding to intermediate density lipoproteins (IDL) was eluted at 91-93 min. Usually there was no detectable material between LDL and HDL peaks. However, if there was a peak or a shoulder to an HDL peak between 100-115 min, the area was digitized and expressed as HDL_1. In cases where the LDL peak was not symmetrical and had a shoulder peak between 90-95 min, the area was digitized as Lp(a).

The recovery of cholesterol in VLDL + LDL by HPLC method was 42 ± 13% as compared to 37 ± 14% by density gradient ultracentrifugation (Table 2). The recovery of VLDL + LDL cholesterol was significantly higher by HPLC than by density gradient ultracentrifugation. The total recovery of cholesterol (91 ± 8% by HPLC vs 90 ± 8% by density gradient ultracentrifugation) or the recovery of cholesterol in HDL (49 ± 10% by HPLC vs 53 ± 9% by density gradient ultracentrifugation) were not significantly different by the two methods. It is possible that the larger volume collected by density gradient ultracentrifugation for the VLDL + LDL fraction may have decreased the sensitivity of the cholesterol, resulting in lower recovery. The apoproteins separated by 3.5% and 10% polyacrylamide sodium dodecyl sulfate gels (10) from these peaks, as described previously (6), were characteristic of these lipoproteins.

Quantitation of plasma lipoprotein profiles. Most plasma lipoproteins were separated by HPLC into three peaks corresponding to VLDL, LDL and HDL as in the

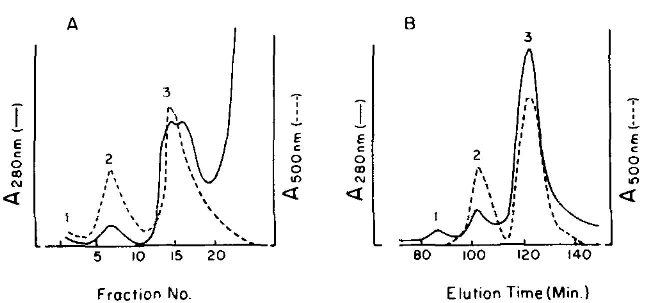

FIG. 1. Distribution of cholesterol (– – –) and protein (——) in lipoproteins separated by density gradient ultracentrifugation (A) and by high performance liquid chromatography using a combination of PW4000 and PW3000 columns (B). Lipoproteins were identified by using standards, density, and apolipoprotein composition. Peaks are labeled 1, very low density lipoproteins; 2, low density lipoproteins; 3, high density lipoproteins. Cholesterol in fractions was measured by the enzymatic method (A500 nm), and proteins were monitored at A280 nm.

TABLE 2

Recovery of Cholesterol in Lipoproteins Separated by Density Gradient Ultracentrifugation and High Performance Liquid Chromatography (HPLC)

		Percent recovery in fractions					
		Separated by density gradient ultracentrifugation			Separated by HPLC		
Animal[a]	Plasma cholesterol (mg/dl)	VLDL + LDL[b,c]	HDL[d]	Total[d]	VLDL + LDL[b]	HDL[d]	Total[d]
1	73	30	59	89	33	51	84
2	96	30	61	91	41	45	86
3	57	23	54	77	28	58	86
4	78	22	64	86	30	56	86
5	189	58	45	103	62	33	95
6	214	52	43	95	54	42	95
7	199	43	46	89	46	60	106
Mean ±S.D.	129 ±68	37 ±14	53 ±9	90 ±8	42 ±13	49 ±10	91 ±8

VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

[a]Animals 1-4 fed the chow diet and 5-7 were fed a high-cholesterol, high-saturated fat diet.

[b]Recoveries of cholesterol in fractions represent percent of plasma cholesterol.

[c]Recovery of cholesterol in VLDL + LDL separated by HPLC was significantly higher ($P < 0.01$) than that by density gradient ultracentrifugation.

[d]Recoveries of cholesterol were not significantly different by the two methods.

case of density gradient ultracentrifugation (Fig. 1). An albumin peak was sometimes seen after the HDL peak. As shown in Figure 2, the areas under the lipoprotein peaks were linearly related to the amount of total protein in lipoprotein standards over a wide range. Areas of most of the lipoprotein peaks in lipoprotein profiles were within the linear range for each lipoprotein. The relationship between the area and the amount of protein in each standard was used to quantitate the amount of protein and expressed as mg/dl plasma.

The protein values measured by this method in LDL and HDL were compared to those measured by the method of Lowry et al. (9). Plasma samples from four animals fed a chow diet and four animals fed the HCHF diet were separated by HPLC and the fractions corresponding to LDL and HDL were pooled. The protein

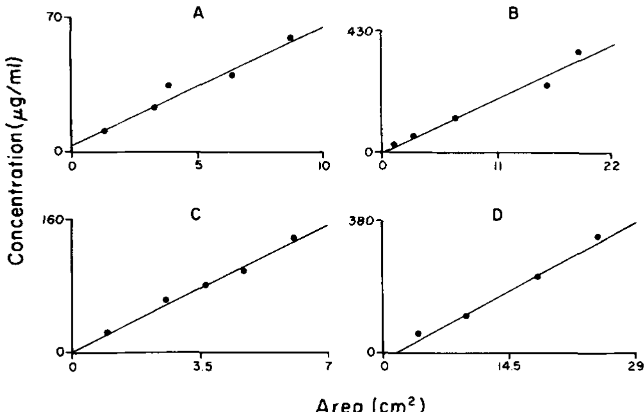

FIG. 2. Relationship between protein concentration and area for lipoprotein standards: A, very low density lipoprotein (VLDL); B, low density lipoprotein (LDL); C, HDL_1 (the unusual lipoprotein separated between LDL and high density lipoprotein [HDL]); and D, HDL. Lines for the standards were drawn by fitting the data by the method of least squares. Correlation coefficients for regression lines for VLDL, LDL, HDL_1 and HDL were 0.984 ($P < 0.01$), 0.968 ($P < 0.01$), 0.990 ($P < 0.01$) and 0.996 ($P < 0.01$), respectively.

contents were measured by the Lowry method (9) and compared with those measured by digitizing. As shown in Table 3, the protein values measured by the two methods were similar.

Lipoprotein profiles of feral baboons. The concentrations of protein in plasma lipoproteins of 96 feral baboons is shown in Table 4. An average plasma lipoprotein protein profile of baboons on the chow diet is characterized by a high concentration of HDL protein (90.9 ± 30.7 mg/dl) with a lesser amount of LDL protein (29.1 ± 13.2 mg/dl) (Table 4). VLDL protein was present in much lower concentrations (8.6 ± 2.6 mg/dl). Smaller concentrations of IDL occasionally seen were not quantitated. These columns did not resolve HDL_2 and HDL_3 completely; however, as reported earlier (6), the major peak of HDL corresponded to HDL_2. Feeding the HCHF diet increased both LDL protein (43.1 ± 21.4 mg/dl) and HDL protein (112.4 ± 34.7 mg/dl) significantly (Table 4). There was no increase in VLDL protein. The distribution of LDL and HDL protein levels in the plasma of the 96 feral baboons consuming chow and also consuming the HCHF diet is given in Figure 3. The LDL protein values varied from 9.2 to 80.1 mg/dl on the chow diet and 11.8 to 121.8 mg/dl on the HCHF diet (Fig. 3). The values for plasma HDL protein varied from 28.8 to 161.92 mg/dl on the chow diet and 29.7 to 194.5 mg/dl on the HCHF diet (Fig. 3).

Plasma lipoprotein profiles of progeny from selected sires. Ninety-seven progeny from three different sire groups were divided on the basis of the responses of the sires' serum cholesterol concentraton to an HCHF diet. The first group of sires had a low response to the HCHF diet (low responders). The second group of sires had a high response to the HCHF (high responders). The third group was a randomly selected group of control sires. Progeny from the control sire group had lipoprotein profiles similar to those in the feral group (Table 4). Progeny of low responders had low levels of HDL on the chow diet (Table 4); however, these values were not significantly different from other groups. There was an increase in their plasma HDL protein upon feeding the HCHF diet (78.4 ± 16.9 mg/dl on chow vs 114.3 ± 31.9 mg/dl on the HCHF diet).

TABLE 3

Comparison of Plasma Lipoprotein Values, Measured by High Performance Liquid Chromatography (HPLC) and Lowry Method, on Chow and High-Cholesterol, High Fat (HCHF) Diets

		LDL (mg/dl plasma)		HDL (mg/dl plasma)	
Diet	Animals	HPLC[a]	Lowry	HPLC[b]	Lowry
Chow	1	21	27	117	108
	2	17	20	112	110
	3	31	33	106	121
	4	19	23	135	152
	Mean ± S.D.	22.0 ± 6.2	25.8 ± 5.6	117.5 ± 12.5	122.8 ± 20.3
HCHF	1	51	46	199	199
	2	23	23	190	198
	3	37	39	140	130
	4	46	53	186	191
	Mean ± S.D.	39.3 ± 12.3	40.3 ± 12.8	178.8 ± 26.4	179.5 ± 33.1

LDL, low density lipoproteins; HDL, high density lipoproteins.

[a]Values for LDL by HPLC and Lowry are not significantly different ($P > 0.05$).

[b]Values for HDL by HPLC and Lowry are not significantly different ($P > 0.05$).

TABLE 4

Lipoprotein Protein Values of Baboons on Chow and High-Cholesterol, High-Fat (HCHF) Diet[a]

Group (n)	Chow diet			HCHF diet		
	VLDL	LDL	HDL	VLDL	LDL	HDL
1. Feral (96)	8.6 ± 2.6[b]	29.1 ± 13.2	90.9 ± 30.7	8.7 ± 6.2	43.1 ± 21.4[e]	112.4 ± 34.7[e]
2. Progeny of low-responding sires (22)	7.9 ± 1.5	29.6 ± 9.0	78.4 ± 16.9	7.5 ± 1.0	31.5 ± 15.2[g]	114.3 ± 31.9[e]
3. Progeny of high-responding sires (55)	7.7 ± 1.5	30.3 ± 9.9	99.3 ± 31.6	8.3 ± 2.8	57.0 ± 28.3[e]	127.6 ± 39.5[e]
4. Progeny of control sires (21)	8.1 ± 0.8	36.0 ± 15.4	90.1 ± 26.3	7.6 ± 1.2	44.4 ± 18.7[e]	128.7 ± 35.2[e]
5. Animals with high HDL_1 (18)	5.2 ± 3.9	30.5 ± 13.4	96.3 ± 27.9 (6.8 ± 3.4)[d]	6.8 ± 3.4	61.4 ± 32.3[e]	113.3 ± 37.3 (44.1 ± 12.2)[d,e]
6. Animals with Lp(a) (17)	6.1 ± 2.5	31.1 ± 7.9 (4.8 ± 2.4)[c]	77.4 ± 26.8	5.4 ± 3.7	48.3 ± 27.7[e] (5.2 ± 2.5)[c]	127.9 ± 48.1[e]
7. Animals with high VLDL (15)	8.2 ± 1.5	33.4 ± 12.2	88.4 ± 18.9	17.1 ± 11.5[e,f]	48.7 ± 24.5[e]	102.3 ± 40.3[e]

VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.
[a]Lipoproteins were measured after separation by HPLC.
[b]Values have been expressed as mg/dl plasma, mean ± S.D.
[c]Values in parentheses are for Lp(a).
[d]Values in parentheses are for HDL_1.
[e]Values for lipoproteins are significantly greater ($P < 0.05$) than in those on the chow diet within the same group.
[f]Values for lipoproteins are significantly greater ($P < 0.05$) than those for other groups on the same diet.
[g]Values for LDL on HCHF diet in group 2 are significantly different from those of groups 3, 5, 6 and 7 on the same diet. LDL values on HCHF diet for groups 1 and 4 are significantly different from those of group 5 on the same diet.

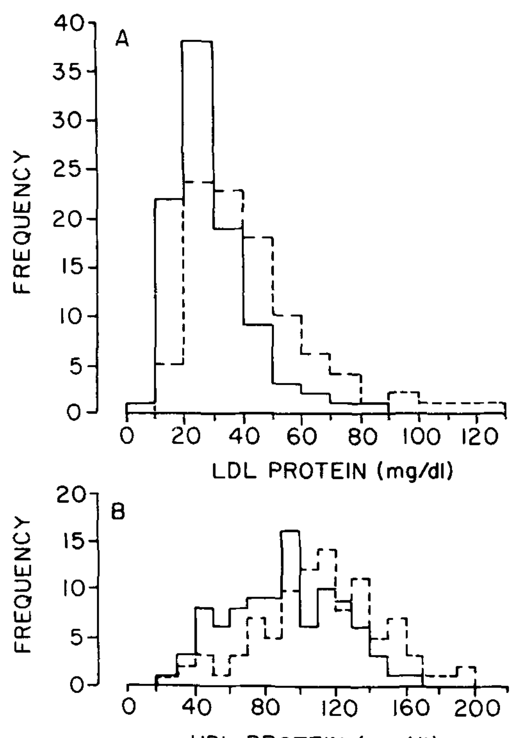

FIG. 3. The distribution of low density lipoprotein (LDL) protein (A) and high density lipoprotein (HDL) protein (B) levels in the plasma of feral baboons (n = 96) on the chow (——) and the high cholesterol high saturated fat diet (– – –).

However, there was very little change in their LDL levels (29.6 ± 9.0 mg/dl on chow vs 31.5 ± 15.2 mg/dl on HCHF diet). On the other hand, progeny of high responders had significant increases in both LDL and HDL levels (Table 4).

The distributions of LDL protein in progeny of low and high responders were similar when they were consuming the chow diet (Fig. 4). However, upon feeding the HCHF diet, LDL distribution changed considerably. The high responding group had a wider distribution of LDL protein (Fig. 4). HDL protein in these animals was also distributed over a wide range on both chow and HCHF diets (Fig. 5). LDL protein values for the high-responding group were significantly higher than for the low-responding group on HCHF diet. However, on the chow diet the values were not different. HDL protein values in the low-responding group were lower than in the high-responding group (78.4 ± 16.9 mg/dl in low responders group vs 99.3 ± 31.6 mg/dl in high responders); however, these values were not significantly different. Upon feeding the HCHF diet, HDL was raised in both groups, and the values between these groups were not significantly different. The control group had a distribution similar to that for the feral group.

A few animals with low LDL levels showed very little change in their LDL levels when challenged with HCHF diet. In contrast, some animals with high LDL protein levels on the chow diet, when challenged with the HCHF diet, raised their LDL protein level considerably, and those values stayed at the higher part of the distribution. The lipoprotein profiles of these animals are given in Figure 6.

Animals with high and low levels of HDL protein were also identified. Lipoprotein patterns of high and low HDL animals are shown in Figure 7.

Unusual lipoprotein patterns. Progeny of a few sires had unusual lipoprotein patterns, with a peak between LDL and HDL (Fig. 7). This lipoprotein was originally described as $F^{\circ}_{1.20}$ 9–28 lipoprotein based on its flotation characteristics by analytical ultracentrifugation (11). Because its physical characteristics and composition are similar to what has been called HDL_1, we have used that term for it (12). This lipoprotein was separated either as a distinct peak between LDL and HDL or as a shoulder to the HDL peak (6). It is seen principally in the plasma of animals consuming the HCHF diet (Fig. 7). The average protein concentration of HDL_1 in animals with this lipoprotein on the HCHF diet was 44.1 ± 12.2 mg/dl. Occasionally this lipoprotein was seen on the chow diet; however, the protein content was very low (Table 4).

Some animals had a lipoprotein pattern in which a small peak preceded the main regular peak of LDL (Fig. 8). Upon density gradient ultracentrifugation, a small peak followed the regular LDL peak (Fig. 8). It appears, therefore, that the small peak detected by HPLC had a higher density but a larger size than LDL. This peak seems to represent Lp(a) (13). The concentration of Lp(a) protein in animals with this lipoprotein peak was estimated to be 4.8 ± 2.4 mg/dl on the chow diet. Cholesterol and saturated fat feeding did not affect the concentration of this lipoprotein. This lipoprotein pattern occurred mainly in the progeny of two sires.

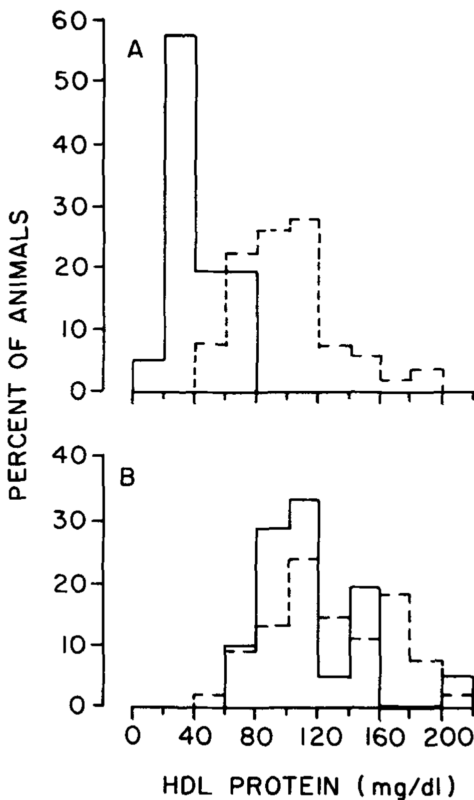

FIG. 5. The distribution of high density lipoprotein (HDL) protein levels in the plasma of pedigreed progeny from low-responding (——) and high-responding (– – –) sires on the chow (A) and the high cholesterol, high saturated fat diet (B).

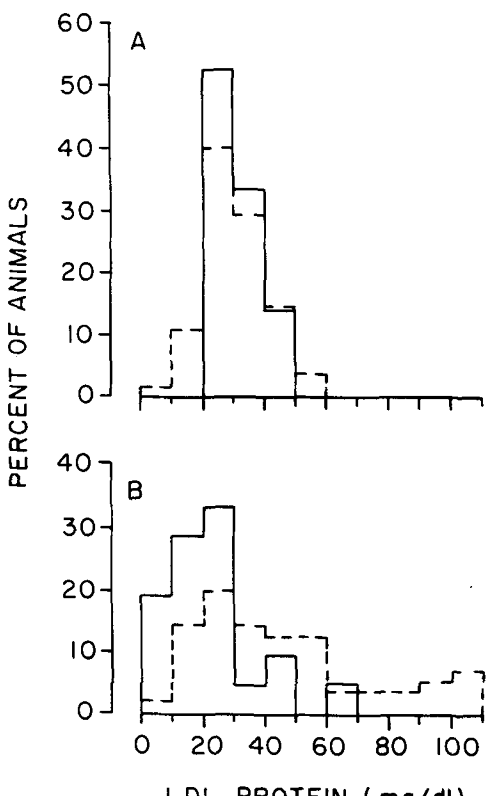

FIG. 4. The distribution of low density lipoprotein (LDL) protein levels in the plasma of pedigreed progeny from low-responding (——) and high-responding (– – –) sires on the chow (A) and the high cholesterol, high saturated fat diet (B).

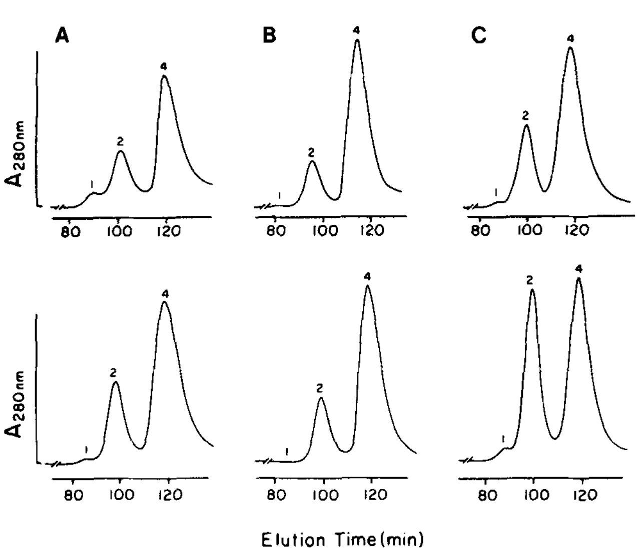

FIG. 6. Lipoprotein patterns separated by high performance liquid chromatography for representative animals showing different phenotypes. Numbers 1, 2 and 4 represent very low density, low density (LDL) and high density lipoproteins, respectively. Lipoprotein patterns A, B and C correspond to average profile, low LDL animals and high LDL animals, respectively. Lipoprotein patterns in the top row are from the chow diet and in the bottom row from the high cholesterol, high saturated fat diet.

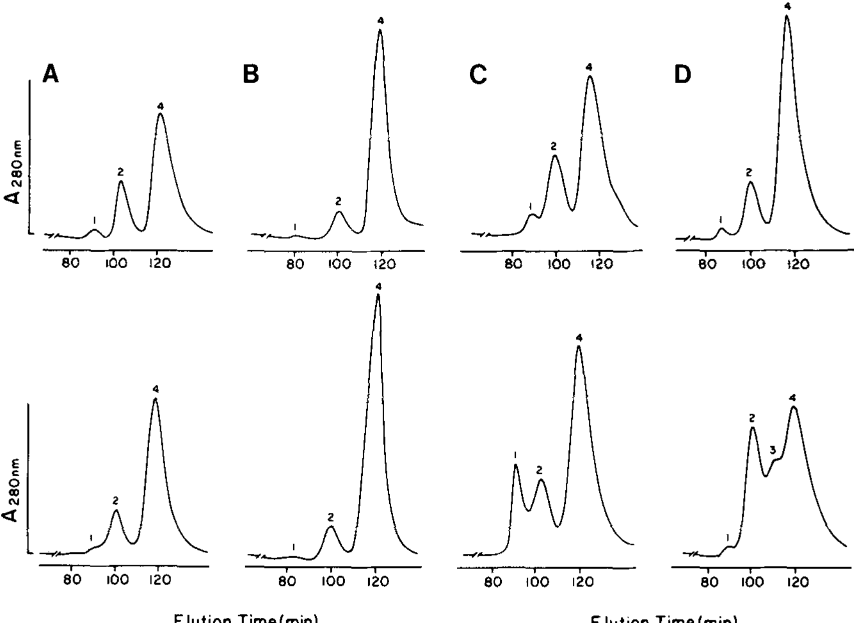

FIG. 7. Lipoprotein patterns separated by high performance liquid chromatography for representative animals showing different phenotypes on chow (top row) and high cholesterol, high saturated fat diet (bottom row). Numbers 1, 2, 3 and 4 correspond to very low density lipoprotein (VLDL), low density lipoprotein (LDL), HDL_1 and high density lipoprotein (HDL), respectively. Phenotypes A, B, C and D correspond to low HDL, high HDL, high VLDL and high LDL with high HDL_1, respectively.

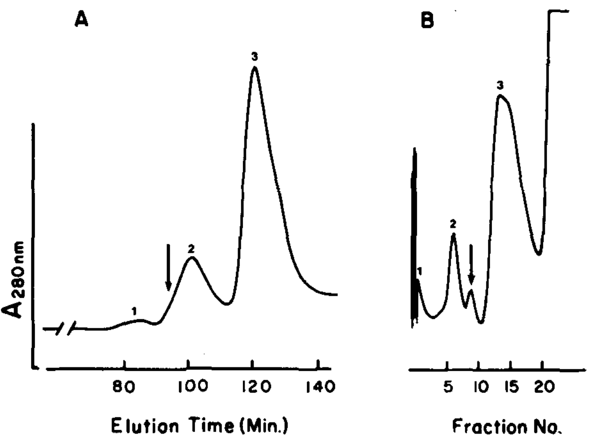

FIG. 8. Lipoproteins separated by high performance liquid chromatography (HPLC) (A) and density gradient ultracentrifugation (B). Numbers 1, 2 and 3 correspond to very low density, low density (LDL) and high density lipoproteins. On separation by HPLC, LDL has a shoulder peak (elution time 90–95 min, denoted with arrow) with larger particles than regular LDL. On separation by density gradient, the second peak of LDL (denoted with arrow) has higher density.

A few animals accumulated relatively high levels of VLDL (Fig. 7) in their plasma when challenged with the HCHF diet (Table 4). The VLDL protein values on the HCHF diet for this group were significantly higher than for other groups. Our studies were not able to determine whether the VLDL was rich in cholesterol or had β-mobility on agarose gel electrophoresis.

DISCUSSION

Comparison with other methods. A number of methods exist for measuring plasma lipoproteins. Most are laborious and time-consuming and require ultracentrifugation (7,13–17), column chromatography (18) or electrophoresis (19–21). The most extensively used technique is that standardized by the Lipid Research Clinics Program (15). According to this method, plasma lipoprotein cholesterol is measured following separation of lipoproteins by ultracentrifugation and precipitation with heparin-manganese chloride. Most of the other methods also measure lipoprotein cholesterol or lipids. Recent studies suggest that apolipoproteins may be better discriminators of atherogenesis than lipids (22–25). The HPLC technique for apolipoproteins is simple, measures total protein (although not individual apolipoproteins) and is convenient for screening a large population. Smaller plasma samples are required, and therefore the technique can be used for the quantification of infant plasma lipoproteins. The technique was validated and applied in a large population of pedigreed baboons. A number of abnormal lipoprotein phenotypes or dyslipoproteinemias were demonstrated.

The recovery of lipoproteins as indicated by total cholesterol in lipoproteins was greater than 90%. The area under the lipoprotein peak detected by absorbance at 280 nm was linearly related to the amount of total protein in each lipoprotein standard over a wide range. Areas under the lipoprotein peaks separated from 50–100 μl plasma were well within this range. Thus, the protein concentrations of lipoproteins were measured accurately, and the protein measured by this method gave values similar to those given by the Lowry method (9) after pooling the

fractions. Using a combination of these columns, the major lipoproteins were resolved; however, the HDL subfractions could not be resolved well.

Application to survey of lipoprotein phenotypes. The lipoprotein profiles of more than 500 pedigreed baboons on two diets (chow and HCHF) were characterized by this method. The lipoprotein phenotype of 96 feral, unselected baboons (the "average" baboon) was characterized by a high concentration of HDL with varying amounts of LDL. VLDL was present in detectable amounts but much less than that in normal human subjects (26). In most cases intermediate density lipoprotein was not detected in plasma lipoproteins. Feeding a diet enriched in cholesterol and saturated fat increased both LDL and HDL, but the increase varied among individuals. Thus, the response to cholesterol diet in baboons seems to be similar to that in humans (27), where both apo-B and apo-A-I were significantly increased by feeding a fat- and cholesterol-rich diet.

This technique was also applied to survey the lipoprotein profiles of pedigreed baboon progeny in three different sire groups. These progeny groups were selected on the basis of the response of sires to the HCHF diet. The groups were low-responding, high-responding and control (unselected). Lipoprotein levels were similar in all three groups on the chow diet. However, response to HCHF diet was different among these groups. Response to HCHF diet in control and high-responding progeny groups was similar to that in the feral group except that the high-responding group had high LDL levels. The low-responding group did not have any increase in LDL levels. All the groups, however, had significant increases in HDL levels. Thus the high- and low-responding groups differed in LDL levels on the HCHF diet, suggesting that there was a modulation of dietary response by the genetic make-up of these animals. Among these groups a few animals showed very little or no change in their LDL when challenged with the HCHF diet. These animals did have a significant increase in their HDL and were called low LDL animals. In contrast, some animals with high LDL on the chow diet had extreme response to HCHF diet in their LDL levels. These animals, like low LDL animals, had increases in their HDL and thus were called high LDL animals. Similarly, animals with high and low HDL without a difference in LDL levels in response to the HCHF diet were also recognized. These genetic animals can be used to study the metabolic differences responsible for differing levels of LDL and HDL.

Several interesting and unusual lipoprotein phenotypes were detected by this HPLC survey of lipoproteins in the pedigreed progeny of the colony. These dyslipoproteinemic patterns differed from the average lipoprotein pattern in several respects. One of the dyslipoproteinemias was characterized by the presence of lipoproteins intermediate to LDL and HDL. These were induced mainly by feeding a diet rich in cholesterol and saturated fat (12) and contained mostly apoproteins A-I and E. They are different from Lp(a) as they do not contain apo-B (11). These lipoproteins are similar to HDL_C (28), but since progeny of only two sires showed HDL_1 on a fat and cholesterol diet, and since in some cases they were present on the chow diet (6), it is unlikely that they are identical to HDL_C. Other dyslipoproteinemias were characterized by the presence of either high or low levels of LDL and HDL without affecting the levels of other lipoproteins. In some animals a distinct peak of Lp(a) was detected. Progeny and sires had similar lipoprotein phenotypes, a finding consistent with the sire effects on serum cholesterol observed by Mott et al. (29) and major gene effects on lipoprotein phenotypes observed by VandeBerg et al. (2).

ACKNOWLEDGMENTS

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Plasma and Lipoprotein Fatty Acid Composition in Glycogen Storage Disease Type I

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Nocturnal intragastric feeding has been shown to be an effective means to improve clinical and biochemical features in glycogen storage disease type I (GSD-I). In this study, we investigated the fatty acid patterns in a whole plasma and in circulating lipoproteins in patients on this therapy. The results demonstrated massive concentration of total fatty acids coupled with higher levels of triglycerides, free cholesterol, cholesterol ester and phospholipids. This hyperlipidemia involved all fatty acids without distinction of carbon or bond numbers. However, the increase was more pronounced for saturated than polyunsaturated fatty acids, as was demonstrated by the ratios of both oleic acid to linoleic acid (1.91 ± 0.40 vs 0.80 ± 0.09 in controls) and of ω3 + ω6 to ω9 fatty acid families (0.92 ± 0.11 vs 1.66 ± 0.08 in controls). The fatty acid patterns in very low (VLDL), low (LDL) and high (HDL) density lipoprotein showed substantial differences in composition, reflecting an association between an abnormal lipoprotein pattern and essential fatty acid deficiency. Furthermore, GSD-I patients exhibited a significant increase in VLDL (17 ± 2 vs 47 ± 7 mg/dl) and LDL cholesterol (124 ± 7 vs 206 ± 24 mg/dl), coupled with a decrease in HDL cholesterol (49 ± 4 vs 28 ± 3 mg/dl). These data documenting high LDL cholesterol and low HDL cholesterol associated with an increased concentration and proportion of saturated fatty acids suggest that GSD-I patients on nocturnal intragastric feeding are at high risk for atherosclerosis and its complications.
Lipids 22, 381-385 (1987).

Von Gierke's disease (glycogen storage disease type I [GSD-I]) is caused by an inherited defect of glucose-6-phosphatase that leads to glycogen accumulation in liver, muscles and kidneys (1). In addition to hepatomegaly, hypoglycemia and failure to thrive, varying degrees of hyperlipidemia and hypercholesterolemia are present in patients with this disorder (2-5).

It has been shown that nocturnal and continuous infusion of glucose and amino acids is helpful and markedly improves metabolic complications and growth (6,7). Although beneficial, the treatment has a limited capacity to return plasma lipids and lipoproteins toward normal. In a previous study, increased very low (VLDL) and low (LDL) density lipoprotein cholesterol and low levels of high density lipoprotein (HDL) cholesterol have remained troublesome features in view of their correlation to cardiovascular disease (8).

On the other hand, this form of treatment could lead to essential fatty acid (EFA) deficiency, because requirements are calculated as a percentage of total energy (9). A similar situation has already been documented in patients with protein-calorie malnutrition treated with hypertonic glucose and amino acids (10-12). Because of elevated pyruvate concentration generated by glycolysis, the synthesis of saturated fatty acids is increased. The strong correlation between saturated fatty acids and the incidence of coronary heart disease calls for careful consideration, in view of our previously documented findings in the same patients (8) and because saturated fat appears to have an effect on cholesterol levels (13) and LDL (14).

The present study was designed to investigate the fatty acid composition in whole plasma and in the major lipoprotein classes. The results could be useful in understanding the metabolic and biochemical alterations in GSD-I patients. Furthermore, the findings could be useful for designing strategies for intervention adapted to both short- and long-term outcomes of GSD-I patients.

MATERIALS AND METHODS

Patients. Six patients (four females and two males, ages between 10 and 22 years) with biopsy-proven GSD-I comprised the subjects. All patients were hospitalized at the time of investigation and had been receiving nocturnal intragastric feeding for 5-6 years. The infusion consisted of one-third of estimated caloric requirements as Vivonex HN from Norwich Eaton (Cambridge, Ontario, Canada), which contains (per liter) 211 g carbohydrate, 43.3 g crystalline amino acids and 0.87 g fat in the form of safflower (75% of polyunsaturated fatty acids [PUFA]). The diurnal diet provided a caloric proportion of 60-70% carbohydrate, 15-20% protein and the rest fat. The follow-up showed an improvement in clinical growth and biochemical parameters (blood glucose levels, lactate, pyruvate, uric acid and SGOT). A substantial decrease was also found in the levels of triglyceride (TG, 45%) and total cholesterol (TC, 29%). Six healthy subjects, matched for age and sex and having ideal body weights, served as controls. Blood was collected on EDTA (1 mg/ml) and sodium azide (0.05%) 4 hr after cessation of nocturnal intragastric feeding.

Isolation of lipoproteins. Lipoprotein fractions were isolated by sequential ultracentrifugation according to Havel et al. (15) with a Ti-50 rotor in a Beckman Model L5-65 ultracentrifuge. VLDL and LDL were prepared at 1.006 g/ml and 1.063 g/ml, respectively, following centrifugation at 100,000 × g for 18 hr at 5 C. The HDL fraction was obtained by adjusting the LDL infranatant to 1.21 g/ml and by centrifuging for 48 hr. Lipoproteins were washed by one additional spin at their respective densities and dialyzed exhaustively against 0.15 M NaCl, 0.001 M EDTA, pH 7.0.

Lipid and lipoprotein determinations. The whole plasma was assayed for TG, TC and free cholesterol (FC) using commercial kits (Boehringer Mannheim, Montreal, Canada). Esterified cholesterol was calculated as the difference between TC and FC. Phospholipids were determined by the method of Bartlett (16). HDL cholesterol was measured after precipitation of VLDL and LDL with

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phosphotungstic acid (17). The assay for LDL cholesterol was performed using polyvinylsulphate (Boehringer Mannheim) (18). VLDL cholesterol was calculated from the difference between cholesterol in the polyvinylsulphate supernatant and HDL cholesterol. Electron microscopy of VLDL particles was performed on a Zeiss EM-10 microscope, using negative staining with 1% phosphotungstic acid (pH 7.2), as described previously (19). The diameter of 500–800 particles was then determined.

Gas liquid chromatography analyses. Fatty acids in whole plasma and in the different lipoprotein fractions were assayed by an improved method recently described by this laboratory (20). Briefly, each sample to be analyzed underwent direct transesterification or specific methylation of free fatty acid (FFA), followed by injection into a HP 5880 gas chromatograph using a 30-m fused silica column wall coated with SP 2330.

Statistical analysis. All data were expressed as mean ± SEM. Statistical differences were assessed by the Student's two-tail t-test. Any statistical significance less than $P < 0.05$ was admissible.

RESULTS

Data in the six GSD-I patients compared to the six age-matched controls showed persistent hypertriglyceridemia (768 ± 190 vs 64 ± 3 mg/dl), hypercholesterolemia (282 ± 32 vs 183 ± 10 mg/dl) and hyperphospholipidemia (472 ± 40 vs 184 ± 5) despite 5–6 yr of nocturnal intragastric feeding (Table 1) and a general improvement of some of the clinical and metabolic manifestations of the disease. VLDL particles were larger (401 ± 17 vs 308 ± 11Å, $P < 0.05$) and contained a higher concentration of TG than controls (625 ± 196 vs 31 ± 4 mg/dl, $P < 0.01$). Cholesterol content of VLDL (47 ± 7 vs 17 ± 2 mg/dl) as well as that of LDL (206 ± 24 vs 124 ± 7 mg/dl) was increased, while that of HDL (28 ± 3 vs 49 ± 4 mg/dl) was decreased. When measured, the percentage of cholesterol ester (CE) was found to be lower in GSD-I (62 ± 3%) than in healthy controls (74 ± 1%).

Table 2 shows that total plasma fatty acids were greatly increased in patients compared to controls. The total increase was close to fourfold and affected all fatty acids. However, the proportion of saturated fatty acids was higher and that of essential unsaturates (linoleic, arachidonic and docosahexaenoic acids) was lower. The relative drop in unsaturates affected the ω3 and ω6 families and led to a decrease in the 18:1ω9/18:2ω6 ratio as well as an increase in the ω3 and ω6 over the ω9 fatty acid family. The 18:2ω6/20:4ω6 ratio also was significantly lower when compared to controls.

When the fatty acid composition of individual lipoprotein fractions was analyzed, it was different from that of controls (Table 3). VLDL and LDL showed a comparable increase of myristic, palmitic and palmitoleic acids along with a proportionate drop in linoleic and docosahexaenoic acids. The HDL fraction showed only a decline in the latter two fatty acids. The 18:1ω9/18:2ω6 ratio was significantly increased in the LDL and HDL fractions. However, only a trend in the same direction appeared in the case of the VLDL, which showed a decrease of oleic acid. The 18:2ω6/20:4ω6 ratios of all lipoprotein classes were decreased.

DISCUSSION

Increased levels of plasma lipids and VLDL and LDL cholesterol with a decline in percentages of both CE and HDL cholesterol have previously been reported in patients with GSD-I following nocturnal intragastric feeding (8). The present study confirms these findings but provides additional information on the profile of plasma and lipoprotein fatty acids. Plasma fatty acids were distinctly increased. However, the pattern of fatty acids was abnormal, in that relative to the saturates and monounsaturates the PUFA were decreased. It should be noted that in the GSD-I patients about 10% of total calories was derived from fat. It is therefore likely that their daily intake of PUFA was below the 5–6% total kcal generally considered desirable (21).

However, these changes could also be due to the well-known increase in lipogenesis from carbohydrates, which constitute the predominant source of calories in GSD-I patients on nocturnal intragastric feeding. This is consonant with the demonstration that liver homogenates from individuals with glucose-6-phosphatase deficiency convert citrate to fatty acids (22). A further argument comes from the observations by Sadeghi-Nijad et al. (23) suggesting that excess hepatic glycolysis causes an increase in hepatic content of NADH, NADPH and acetyl CoA, three compounds important in fatty acid and cholesterol synthesis (1). Therefore, it is likely that an

TABLE 1

Plasma Lipids and Lipoprotein Cholesterol

	Plasma					Cholesterol		
	TG	PL	TC	FC	CE as % of TC	VLDL	LDL	HDL
Controls	64 ± 3	184 ± 5	183 ± 10	48 ± 3	74 ± 1	17 ± 2	124 ± 7	49 ± 4
GSD-I	768 ± 190[a]	472 ± 40[a]	282 ± 32[b]	109 ± 19[b]	62 ± 3[c]	47 ± 7[a]	206 ± 24[b]	28 ± 3[a]

Values are mean ±SEM and expressed as mg/dl of plasma. TG, triglyceride; PL, phospholipid; TC, total cholesterol; FC, free cholesterol; CE, cholesterol ester; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; GSD-I, glycogen storage disease type I.

[a] $p < 0.005$.

[b] $p < 0.02$.

[c] $p < 0.01$.

TABLE 2

Plasma Fatty Acid Concentrations and Percentage Composition in Glycogen Storage Disease Type I (GSD-I) Patients and Healthy Subjects

Fatty acid	Fatty acid concentrations (μg/ml)		Fatty acid percentage	
	Controls	GSD-I	Controls	GSD-I
14:0	23.0 ± 1.8	248.0 ± 63.8[a]	1.0 ± 0.1	2.5 ± 0.2[b]
15:0	8.4 ± 0.6		0.4 ± 0.1	
16:0	510.3 ± 24.0	3066.0 ± 642.2[c]	21.4 ± 0.5	32.5 ± 0.8[b]
16:1ω7	33.3 ± 7.5	667.5 ± 146.5[c]	1.4 ± 0.3	7.3 ± 0.9[b]
18:0	184.2 ± 9.6	572.1 ± 116.7[a]	7.7 ± 0.1	6.2 ± 0.3[a]
18:1ω9	584.2 ± 27.4	2434.2 ± 468.6[a]	24.6 ± 0.9	26.2 ± 1.4
18:2ω6	734.1 ± 41.4	1505.9 ± 398.0	30.8 ± 0.7	16.0 ± 2.3[b]
20:3ω6	35.4 ± 2.6	145.1 ± 21.5[c]	1.5 ± 0.1	1.7 ± 0.2
20:4ω6	203.8 ± 18.4	482.3 ± 108.1[d]	8.5 ± 0.5	5.3 ± 0.5[c]
22:6ω3	42.9 ± 7.5	63.5 ± 8.1	1.8 ± 0.2	0.9 ± 0.1[a]
24:1ω9	19.4 ± 1.4	58.1 ± 34.3	0.8 ± 0.1	0.5 ± 0.2
Total	2381.5 ± 109.8	9377.4 ± 1893.8[c]		
Family				
ω3			1.8 ± 0.2	1.1 ± 0.1[d]
ω6			40.8 ± 0.6	22.9 ± 2.7[b]
ω9			25.4 ± 0.9	27.2 ± 1.6
Ratio				
18:1ω9/18:2ω6			0.80 ± 0.04	1.91 ± 0.40[d]
18:2ω6/20:4ω6			3.71 ± 0.35	2.65 ± 0.24[d]
(ω3 + ω6)/ω9			1.66 ± 0.08	0.92 ± 0.11[a]
Nonsaturated			69.4 ± 0.4	58.5 ± 1.2[b]

GSD-I group consisted of six patients who were compared with a group of six healthy controls. Fatty acid percentage is expressed as percentage of the total amount of fatty acids present. Fatty acids contributing less than 0.2% of the total have been omitted from the table. Student's t-test (two-tailed) was used to compare differences between means.

[a] $p < 0.01$.
[b] $p < 0.001$.
[c] $p < 0.005$.
[d] $p < 0.05$.

TABLE 3

Fatty Acid Composition of Lipoprotein Fractions Expressed as a Percentage of Each Fraction

Fatty acid	VLDL		LDL		HDL	
	Control	GSD-I	Control	GSD-I	Control	GSD-I
14:0	1.7 ± 0.2	3.5 ± 0.3[a]	0.8 ± 0.1	2.7 ± 0.7[a]	0.6 ± 0.1	1.4 ± 0.1[b]
15:0	0.5 ± 0.1		0.3 ± 0.1		0.3 ± 0.1	
16:0	25.8 ± 0.9	36.1 ± 0.9[b]	18.7 ± 0.4	29.4 ± 0.9[b]	20.4 ± 0.5	28.8 ± 0.7[b]
16:1ω7	2.9 ± 0.3	7.8 ± 0.9[b]	1.6 ± 0.2	7.0 ± 0.8[b]	1.4 ± 0.1	4.8 ± 1.0[a]
18:0	5.4 ± 0.4	5.6 ± 0.3	6.0 ± 0.1	6.4 ± 0.3	8.7 ± 0.1	9.0 ± 0.1
18:1ω9	39.2 ± 1.0	28.4 ± 1.7[b]	23.0 ± 0.8	23.9 ± 1.8	18.5 ± 0.7	19.3 ± 1.1
18:2ω6	18.9 ± 0.8	13.2 ± 2.3[a]	35.7 ± 0.8	17.9 ± 1.4[b]	31.0 ± 0.7	20.3 ± 2.2[c]
18:3ω3					0.4 ± 0.1	0.3
20:0			0.3 ± 0.1	2.0	0.3 ± 0.1	0.2
20:3ω6			1.3 ± 0.1	1.8 ± 0.1[a]	2.0 ± 0.1	3.6 ± 0.3[b]
20:4ω6	3.2 ± 0.2	3.5 ± 0.3	8.0 ± 0.7	6.5 ± 0.5	10.9 ± 0.7	9.9 ± 0.3
22:1ω9			0.5 ± 0.1	0.4		
22:6ω3	1.4 ± 0.2	0.7 ± 0.1[a]	1.8 ± 0.2	0.7 ± 0.1[d]	3.1 ± 0.4	1.5 ± 0.2[c]
24:1ω9	0.8 ± 0.1	0.7	1.7 ± 0.1	1.3 ± 0.6	1.8 ± 0.1	0.6 ± 0.1[b]
Family						
ω3	1.8 ± 0.4	0.9 ± 0.2	2.2 ± 0.3	0.8 ± 0.1[b]	3.3 ± 0.4	2.3 ± 0.4
ω6	22.1 ± 0.8	17.8 ± 2.5	44.9 ± 0.3	25.9 ± 1.7[b]	44.0 ± 0.4	33.8 ± 2.4[c]
ω9	39.8 ± 0.9	28.5 ± 1.6[b]	25.2 ± 0.7	26.4 ± 1.0	21.0 ± 0.6	19.9 ± 1.1
Ratio						
18:1ω9/18:2ω6	2.09 ± 0.11	2.60 ± 0.56	0.64 ± 0.02	1.36 ± 0.16[c]	0.60 ± 0.01	1.02 ± 0.15[a]
18:2ω6/20:4ω6	6.12 ± 0.55	3.69 ± 0.52[d]	4.67 ± 0.56	2.84 ± 0.49[d]	2.92 ± 0.29	2.06 ± 0.21[a]
(ω3 + ω6)/ω9	0.60 ± 0.03	0.68 ± 0.13	1.88 ± 0.08	1.02 ± 0.08[b]	2.38 ± 0.14	1.89 ± 0.27[c]
Nonsaturated	66.6 ± 0.9	54.7 ± 1.5[b]	73.9 ± 0.3	60.2 ± 1.8[b]	69.7 ± 0.4	60.8 ± 0.7[b]

VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; GSD-I, glycogen storage disease type I. Individual plasma lipoprotein classes were isolated by sequential ultracentrifugation. VLDL was separated at 1.006 g/ml, LDL at 1.063 g/ml and HDL at 1.21 g/ml. The fractions were washed and dialyzed before gas liquid chromatographic analyses. The purity of the different lipoprotein classes was checked by agarose gel electrophoresis. Fatty acids contributing less than 0.2% of the total have been omitted from the table.

[a] $p < 0.05$.
[b] $p < .001$.
[c] $p < .005$.
[d] $p < 0.01$.

increase in glycerol and acetyl CoA generated from the glycolytic pathway, together with high levels of reduced cofactors, could sustain an increased rate of TG and lipoprotein synthesis. Thus de novo synthesis results in an increased concentration of saturates and monounsaturates relative to the polyunsaturates which cannot be produced endogenously. The biochemical criteria for EFA deficiency, i.e., increased 18:1/18:2 ratio coupled with an increase in the ω3 and ω6/ω9 families, are well established. The patients studied met these criteria for EFA deficiency, as the ratios observed were comparable to those previously reported (24-26).

Even though there was no clinical evidence of EFA deficiency, the relative decrease in PUFA could be responsible for some of the anomalies observed in lipid profiles of these patients: i) The imbalance reflected by the abnormal ratio of nonessential/essential fatty acids could affect the composition, interconversion and clearance of the major lipoprotein fractions. For instance, the lipoprotein lipase (LPL) substrate interaction could be prejudiced in view of observations showing a high affinity of LPL for TG made up of PUFA (27). In our previous investigation (8), we demonstrated that the catabolism of plasma TG is impaired in this disorder. LPL activity was also substantially reduced in patients. Whether the mechanism of hypertriglyceridemia in these patients is related to an inhibitory factor or to the excessive amount of saturated fatty acids remains to be investigated.

ii) The recovery of TG-derived fatty acids in peripheral tissues as mirrored by the FFA fraction shows a predominance of saturated fatty acids because tissues in GSD-I patients probably contain an inordinate proportion of saturates and monounsaturates. Had membrane composition been examined, it is possible that a relative deficiency of EFA could have been documented, since there is ample evidence that EFA deficiency may lead to alterations in PUFA profile of tissue lipids (28,29). More important is the fact that such changes are known to be associated with alterations of structure and function (30).

iii) Lecithin cholesterol acyltransferase (LCAT), the second lipoprotein-related enzyme, uses mainly HDL as a substrate, translocating linoleic acid at the 2-position of lecithin to FC. Regarding the normal main product of the LCAT reaction in plasma, cholesteryl linoleate is the most abundant (31). LCAT also necessitates apo A-I as a cofactor for its activity (31). Since the latter apoprotein was found to be normal in concentration (8), we therefore suggest that EFA may be a limiting factor for the cholesterol esterification in view of its low level (Table 1). Therefore, CE transfer from HDL to VLDL and to LDL might be also affected because of the reduced capacity of these patients to esterify (8).

It is well known that unsaturated fatty acids are desaturated and elongated in endoplasmic reticulum membranes of many tissues (32). The reactions ending in 20:4 from 18:2 are $9,12\text{-}18{:}2 \xrightarrow{\Delta 6} 6,9,12\text{-}18{:}3 \rightarrow 20{:}3 \xrightarrow{\Delta 5} 20{:}4$ and involve Δ6 and Δ5-desaturases. The first enzyme in the chain has a key position and a regulatory function. Brenner has demonstrated that its activity can be modified by dietary and hormonal changes (32). For instance, glucose refeeding, a hyperprotein diet and insulin administration reactivate the enzyme (32). Thus, in our patients, the possible relationship between nocturnal intragastric feeding combined with frequent daytime feeding and Δ6-desaturase activity could explain the lower ratio of 18:2ω6/20:4ω6, which provides an estimate for elongation and desaturation steps. Thus, the much lower values observed suggest that the elongation and desaturation processes are increased in GSD-I patients. A decreased growth rate is one of the clinical features associated with EFA deficiency (33) and, according to Holman, a decrease in growth could postpone the appearance of deficiency (34). Accordingly, this suggests that the impaired growth of GSD-I patients could be due in part to a relative degree of EFA deficiency. On the basis of our observations, it is suggested that the administration of PUFA to these subjects might lead to improved growth.

Hyperlipidemia, as seen in GSD-I patients, may be an important risk factor for heart disease. At this time, we have not detected any clinical indications of coronary artery disease in our young patients. Atherosclerotic changes have, however, been observed in arteries of young asymptomatic children growing up in industrialized countries (35). Therefore, particular attention should be given to all children with hyperlipidemia. Recently, the American Heart Association Committee recommended a "prudent modification of diet" even for healthy children and adolescents as a means of influencing the plasma lipids. Numerous reports have confirmed the effectiveness of PUFA diets in lowering hyperlipidemia (36-38). All studies were uniform in reporting a favorable trend toward decreased LDL cholesterol. Other studies described a fall in the plasma TG (39) and an increase of PUFA percentage associated with lipoproteins (40,41). Since there is a well-known association of saturated fatty acids (42), increased LDL (43), elevated plasma TG (44) and a low HDL level (43) with increased prevalence of coronary heart disease, it seems appropriate to treat our patients who also present these biochemical risk factors with a diet enriched in polyunsaturated fat.

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Growth Support and Metabolism of Phytosterols in *Paramecium tetraurelia*

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The basis of the growth requirement of *Paramecium* for one of several structurally similar phytosterols is not known. Previous research has indicated that selective esterification of only growth-promoting sterols may be a key. In this study, it was found that under certain conditions sterols that fail to support growth (e.g., cholesterol) can be esterified in large amounts in *Paramecium*. We found no compelling evidence to support the hypothesis that steryl esters serve a specialized role in the fatty acid metabolism of the cell. Octadecenoic acid, essential for cell growth, was the major fatty acid in both steryl esters and triglycerides. It was also shown that *P. tetraurelia* can dehydrogenate Δ^0 and Δ^7, as well as Δ^5-3β-hydroxy sterols, to yield the conjugated 5,7-diene derivative. These results indicate the presence of a Δ^5, in addition to a Δ^7, desaturase of the sterol nucleus in this ciliate. Two C_{24} α-ethyl sterols, Δ^{22}-stigmastenol (Δ^{22}) and stigmastanol (Δ^0), were shown for the first time to promote growth. Finally, we found that non-growth-promoting sterols may compose a high percentage of the free sterols of the surface membrane without adversely affecting cell growth or viability. These data support the conclusion that the growth requirement for select phytosterols in *Paramecium* does not involve the structural or functional role of "bulk" sterols in cell membranes.
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The growth requirement for a phytosterol in the ciliated protozoan *Paramecium aurelia* was determined 30 years ago by Conner et al. (1,2). This sterol requirement was subsequently shown to be highly specific. Only a small group of structurally similar phytosterols was found to support growth of this organism in an axenic medium (3). Alkyl substitution (usually ethylation) at C-24 of the sterol side chain was shown to be essential for nutritional activity, and desaturation at C-22,23 of the side chain was shown to enhance activity. Epimerization of the ethyl group at C-24 had no effect.

The basis of auxotrophy for select phytosterols in *Paramecium* is unknown. One hypothesis, forwarded by Conner et al. (4), is that sterol esterification is somehow requisite for cell growth. This proposal was based on a study comparing the metabolism of the growth-promoting sterol stigmasterol with that of the non-growth-promoting sterol cholesterol in *P. aurelia.* It was shown that although both of these Δ^5 sterols were dehydrogenated at the 7,8 position to yield their 5,7-diene derivatives, no cholesterol or 7-dehydrocholesterol was incorporated into the steryl ester (SE) pool. Thus, the data indicated that only growth-promoting 3β-hydroxy sterols are esterified to fatty acids.

In this study, we have expanded the scope of the investigation by Conner et al. (4) to encompass the promotion of growth by and metabolism of a variety of phytosterols in *P. tetraurelia* in both monoxenic and axenic culture. The monoxenic medium used in these experiments was devised to eliminate the problem of contaminant sterols in the crude axenic and monoxenic cerophyl media customarily used for culture of *Paramecium* (5). The axenic medium used in this study was essentially the same as that used by Conner et al. (4), with the exception that cholesterol present in the 1-α-cephalin component was removed in only select series of experiments. Several dramatic quantitative and qualitative differences were found in the growth support and metabolism of phytosterols in monoxenic vs axenic cultures of *P. tetraurelia,* which prompted us to report results with both types of media.

We have tested the hypothesis linking sterol esterification with growth support by comparing the sterol composition of SE from axenic cultures of *Paramecium* with or without cholesterol and supplemented with one of six growth-promoting phytosterols. In addition, the fatty acid composition of SE and triglycerides (TG) has been compared for *P. tetraurelia* cultures supplemented with different phytosterols. This segment of the study was carried out to pursue the possibility that SE perform some specialized function in the fatty acid metabolism of *Paramecium,* rather than serving merely as a nontoxic reserve of sterols and free fatty acids. Also, the question of why the 3-keto steroid $\Delta^{4,22}$-stigmastadienone supports growth of *Paramecium* (3), particularly in light of the sterol esterification hypothesis, has been addressed. Finally, several experiments were performed to determine the extent to which non-growth-promoting sterols can replace stigmasterol and 7-dehydrostigmasterol in the ciliary membrane of *Paramecium.*

MATERIALS AND METHODS

Monoxenic cultures. Initial studies of sterol growth support, uptake and metabolism in *Paramecium* were performed by adding the appropriate sterol(s), sterol derivative or keto-steroid in an ethanolic solution to 3-(N-morpholino)propanesulfonic acid (MOPS) basal medium, autoclaving for 40 min and subsequently adding a dense suspension of *Escherichia coli* B prior to inoculation with *Paramecium* (6). (The concentration of phytosterols added to monoxenic cultures in these experiments, ≅1–5 μg/ml, was well above their solubility in water. Sterols added in ethanolic solution to room temperature MOPS basal medium in this concentration range immediately precipitated, forming large particulate aggregates. Upon autoclaving, the sterols were dispersed as a suspension of fine particles.) MOPS medium consisted of the following: 15 mM MOPS, 3 mM KCl, 1 mM $CaCl_2$, KOH to pH 6.2 plus NaOH to pH 6.5. *E. coli* cultures were grown in 0.4% minimal glucose medium (6) (potassium phosphates plus salts and 20% glucose solutions autoclaved separately).

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Abbreviations: FAME, fatty acyl methyl ester; GLC, gas liquid chromatography; MOPS, 3-(N-morpholino)propanesulfonic acid; SE, steryl ester; TG, triglyceride; TLC, thin layer chromatography.

Flask cultures (1 l total volume) were started with a 0.5% v/v inoculum and grown to early stationary phase (20–24 hr; $A_{650\ nm} \cong 1.8$) on a gyratory shaker at 28 C. Bacteria were harvested by centrifugation for 5 min at 6,500 × g in sterilized Corex bottles, washed once in cold sterile MOPS, recentrifuged and resuspended in a few milliliters of sterile MOPS medium. The dense *E. coli* suspension derived from 1 l of medium was evenly distributed in flasks containing sterile MOPS medium plus sterol (2 l total volume), which were then inoculated at 100–200 cells/ml with *P. tetraurelia* wild-type 51S from an early stationary phase MOPS plus *E. coli* culture (5 μg/ml stigmasterol) and incubated at 25 C.

Control experiments were carried out to determine if steroids added to MOPS basal medium were altered during autoclaving or metabolized by *E. coli.* Stigmasterol, stigmasteryl acetate, stigmastadienone, cholesterol or cholesteryl myristate was added to 5 μg/ml to two sets of 250-ml flasks containing 40 ml of MOPS medium. After autoclaving, one set of flasks was immediately extracted with hexane/ether (1:1, v/v). A dense suspension of *E. coli* was added to the second set of flasks and allowed to incubate at 28 C for 24 hr. Following incubation, the bacteria were harvested by centrifugation and extracted with $CHCl_3$-MeOH (2:1, v/v). The MOPS medium supernatant was also extracted with hexane/ether (1:1, v/v). The following briefly summarizes the results: There was no evidence of metabolism of any of the steroid supplements by *E. coli.* Neither stigmasteryl acetate nor cholesteryl myristate was hydrolyzed during autoclaving or during incubation with the bacteria. However, 20–50% of the recovered steroid was associated with the bacterial pellet, even after washing and recentrifugation. This suggests that a substantial fraction of the steroid supplemented to the medium was taken up by, or adsorbed to, *E. coli.* Also, impurities detected by gas liquid chromatography (GLC) indicated that for both cholesterol and stigmastadienone, ≅10–20% of the added steroid was degraded (perhaps oxygenated) during autoclaving.

Axenic cultures. In the second study of growth support and metabolism of phytosterols in *P. tetraurelia,* the wild-type strain 51S was cultured in Soldo's crude axenic medium (7). This is a proteose and trypticase peptone plus yeast nucleic acid–based medium, rich in added vitamins and high in free fatty acids (≅45 μg/ml), and includes phosphatidylethanolamine (1-α-cephalin) of high polyunsaturated fatty acid content. Sterol was added at 2–5 μg/ml in an ethanolic solution (final [ethanol] ≤ 0.1%) prior to autoclaving. This medium contained ≥2 μg/ml cholesterol, a contaminant of the 1-α-cephalin component (4) (Sigma Chemical Co., St. Louis, Missouri). In the final series of experiments, the 1-α-cephalin was purified (>90% of the cholesterol removed) by silicic acid column chromatography (8,9) prior to addition to the axenic medium. Axenic cultures were inoculated at ≅100 cells/ml from a mid-log phase culture supplemented with 5 μg/ml stigmasterol (inoculum ≤ 2% total culture volume) and incubated at 25 C.

Cell harvest, deciliation and lipid extraction. Monoxenic and axenic cultures of *P. tetraurelia* (51S) were harvested by centrifugation in pear-shaped oil-testing centrifuge tubes at 250 × g for 2 min. Monoxenic cultures were harvested at early stationary phase (70–80 hr growth; nearly cleared of *E. coli*; 0.9–1.2 × 10^4 cells/ml), while axenic cultures were harvested at mid-log phase (70–80 hr growth; 6–8 × 10^3 cells/ml). The cell pellet was washed twice with MOPS basal medium (25 C) prior to deciliation or lipid extraction of whole cells. Cells from monoxenic cultures were deciliated by the calcium shock procedure described by Adoutte et al. (10), except that MOPS basal medium was used in place of Dryl's salt solution. Total lipid was extracted from whole cells or cilia by the method of Folch et al. (11). The chloroform/methanol (2:1, v/v) extraction was carried out in screw cap centrifuge tubes under N_2, in dim light. Butylated hydroxytoluene was added to the solvent mixture at 15 μg/ml to limit oxidation of lipids. All organic solvents were redistilled in glass.

Lipid fractionation and analysis. Total lipids extracted from *Paramecium* grown in either monoxenic or axenic culture were fractionated by silicic acid column chromatography in two steps: (i) A 1.5-ml slurry of 100–200 mesh Bio-Sil A (Bio-Rad, Richmond, California) in $CHCl_3$ was loaded into a 5¾" Pasteur pipet column plugged with glass wool. The column was washed with 5 ml of $CHCl_3$, and the lipid extract was applied in 0.5 ml $CHCl_3$. Neutral lipids were eluted with 6 ml $CHCl_3$, dried under a stream of N_2 and redissolved in 0.5 ml hexane (8,9). (ii) A second Pasteur pipet column was loaded with a 1.5-ml slurry of silicic acid in hexane. Neutral lipids were applied to the column and fractionated with a step gradient of hexane/diethyl ether (8,9). Hydrocarbons and steryl esters were eluted with 100% hexane (3 ml), triglycerides with hexane/ether (99:1, v/v) (6 ml), and fatty acids and alcohols with hexane/ether (95:5, v/v) (4 ml). The column was then stripped with $CHCl_3$ (5 ml) to yield the free sterol fraction (plus small amounts of mono- and diglycerides). The lipid composition of the step-gradient column fractions was examined by thin layer chromatography (TLC) on 250 μ silica gel 60, 20 × 20 cm glass TLC plates (Merck, Rahway, New Jersey) developed in hexane/diethyl ether/formic acid (80:20:2, v/v/v) and visualized with $FeCl_3$ spray reagent (9,12). Lipid fractions were dissolved in $CHCl_3$ and stored at −20 C under N_2 for no more than one week prior to sterol and fatty acid analysis.

The free sterol content of *Paramecium* from both monoxenic and axenic cultures was generally in the range of 40–80 μg per 10^6 cells. Free sterols and other steroids from the neutral lipids of *Paramecium* were tentatively identified by their GLC retention times relative to known standards. The instruments used for this analysis were a Varian 3700 gas chromatograph in tandem with a CDS-111 integrator. Sterols were separated on a 3 ft, 2 mm id glass coil column packed with 3% OV 17 on 100/120 mesh Supelcoport (Supelco, Bellefonte, Pennsylvania). The N_2 flow rate was 20 cc/min, the oven temperature was 260 C (isothermal) and the injector and detector temperatures were 320 C and 350 C, respectively.

More rigorous identification of sterol metabolites from *Paramecium* entailed the following: conversion of 3β-hydroxy sterols to steryl acetates with acetic anhydride (13); GLC analysis of steryl acetates (GLC retention times increased relative to free sterols); separation of steryl acetates according to the number of double bonds in the ring system and alkyl side chain by elution from a column of silicic acid impregnated with 20% $AgNO_3$ (w/w) using a step gradient of hexane/benzene (14); GLC

analysis of steryl acetates in the column fractions; and UV absorbance and mass spectra of steryl acetates and/or free sterols using a Gilford UV-VIS microprocessor-controlled System 2600 spectrophotometer and an AEI Model MS9 mass spectrometer, respectively. UV spectra were indicative of the presence or absence of conjugated double bonds in the B ring at the 5,6 and 7,8 positions. The $\Delta^{5,7}$ sterols have characteristic absorbance maxima in the UV at 271, 281.5 and 293 nm (15). Identification of sterols and steryl acetates by mass spectrometry was based on the M^+ ion (or M^+-60 for steryl acetates), as well as a comparison of the mass numbers and proportions of the major mass fragments with values reported in the literature (16,17). A combination of these methods (argentation column chromatography, UV spectrophotometry and mass spectrometry) was used to help determine the sterol composition of a given free or esterified sterol fraction whenever GLC alone was inadequate. For example, spinasterol ($\Delta^{7,22}$) and 7-dehydrostigmasterol ($\Delta^{5,7,22}$) had very close GLC retention times, but UV absorbance and mass spectra clearly showed that most of the former was dehydrogenated to yield the latter in both axenic and monoxenic cultures.

Fatty acids of steryl esters and triglycerides were transesterified to fatty acyl methyl esters (FAME) for GLC analysis by reacting with 0.5 N sodium methoxide in dry methanol as described by Luddy et al. (18). This reaction also liberated free sterols from steryl esters, which could then be analyzed by GLC. Gas chromatography of FAME was carried out on a 6 ft, 2 mm id glass coil column packed with 10% SP2330 on 100/120 mesh Chromosorb (Supelco). The N_2 flow rate was 20 cc/min, the column oven temperature was 190 C (isothermal) and the injector and detector temperatures were 250 C and 300 C, respectively. Identification of FAME from SE and TG of *Paramecium* was based on comparison of GLC retention times with those of known standards purchased from Sigma and Supelco and generated from total lipids of *E. coli* B. Identification was substantiated or expanded using two methods. The first was separation of subclasses of FAME according to the number of double bonds on 10% (w/w) $AgNO_3$-impregnated TLC plates (250 μ silica gel 60, 5 × 20 cm) developed with hexane/diethyl ether (90:10, v/v) (to separate saturated, mono- and dienoic) or 40:60 (v/v) (to separate tri- and tetraenoic) (9). The FAME subclasses were then scraped from the TLC plates, eluted from the silica gel and analyzed by GLC. The second method was saturation of all double bonds by vortexing a hexane solution of FAME sealed under an atmosphere of H_2 in the presence of 5% paladium catalyst on charcoal (Amend Drug and Chemical, New York, New York), followed by GLC analysis (9). These two methods gave a good estimation of the ratio of 16, 18 and 20 carbon fatty acids, and resolved the FAME pairs 17:cyc/16:2 and 19:cyc/18:2, which otherwise were not separated by GLC. (The 17- and 19-carbon cyclopropane fatty acids were derived from *E. coli* [19], and thus were present only in monoxenic cultures of *Paramecium*.)

Commercial and natural sources of sterols, sterol derivatives and steroids. Table 1 lists the source and

TABLE 1

Sterols, Steryl Acetates, Steryl Esters and Steroids: Sources and Purity

	Source	Purity[a] (%)	Major contaminant
Sterols			
Campesterol [(24R)-ergost-5-en-3β-ol]	Sigma Chem. Co.	99	None
Cholesterol [cholest-5-en-3β-ol]	Sigma Chem. Co.	99	None
Lanosterol [lanosta-8,24-dien-3β-ol]	Sigma Chem. Co.	≅70	Unknown
Stigmastanol	Sigma Chem. Co.	70	Campestanol (30%)
Stigmasterol I [stigmasta-5,22E-dien-3β-ol]	Sigma Chem. Co.	92	Sitosterol (5%)
Stigmasterol II	Applied Science Labs	98	None
Sitosterol [stigmast-5-en-3β-ol]	Applied Science Labs	97	Campesterol (2%)
Fucosterol [(24E)-stigmasta-5,24(28)-dien-3β-ol]	Steraloids, Inc.	99	None
Ergosterol [ergosta-5,7,22E-trien-3β-ol]	Aldrich Chem. Co.	98	None
Δ^{22}-Stigmastenol [stigmast-22E-en-3β-ol]	*Dictyostelium discoideum*	92	Stigmastanol (8%)
Spinasterol [stigmasta-7,22E-dien-3β-ol]	Spinach leaves	70	Δ^{7}-Stigmastenol (20%)
Brassicasterol [ergosta-5,22E-dien-3β-ol]	*Brassica rapa* seed	94	Sitosterol (4%)
Steryl acetates			
Stigmasteryl acetate	Sigma Chem. Co.[b]	96	Sitosteryl acetate (3%)
Ergosteryl acetate	Aldrich Chem. Co.[b]	99	None
Steryl esters			
Cholesteryl myristate	Sigma Chem. Co.	99	None
Cholesteryl oleate	Sigma Chem. Co.	99	None
Keto-steroid			
$\Delta^{4,22}$-Stigmastadienone [stigmasta-4,22E-dien-3-one]	Sigma Chem. Co.	96	Unknown (4%)
Triterpenoid			
Tetrahymanol[c]	*Tetrahymena pyriformis*	99	None

[a]Purity determined by gas liquid chromatography.

[b]Free sterol acetylated via reaction with acetic anhydride.

[c]4,4′-Dimethyl pentacyclic triterpenoid alcohol.

purity of the various sterols, steryl esters, steryl acetates and the 3-keto steroid used in this study. Commercially acquired sterols were recrystallized twice from 100% ethanol. Purity was determined by GLC. The 5-dihydro derivative of stigmasterol, Δ^{22}-stigmastenol, and the pentacyclic triterpenoid alcohol tetrahymanol were isolated from amoebae of *Dictyostelium discoideum* (20) and cells of *Tetrahymena pyriformis* (21), respectively. These two compounds were purified by the following series of procedures. (i) Total lipids were extracted from washed cell pellets with chloroform/methanol (2:1, v/v) (11). (ii) The neutral lipid fraction was eluted from a silicic acid column with five column volumes of $CHCl_3$. (iii) After drying on a rotary evaporator, neutral lipids were saponified in 1 N KOH (95% ethanol) in a sealed culture tube under N_2 at 80 C for 2 hr, and the nonsaponifiables were extracted twice with hexane (9). (iv) The hexane extract was dried on a rotary evaporator and the sterols or triterpenoids were recrystallized twice from hot ethanol.

The phytosterols spinasterol ($\Delta^{7,22}$-stigmastadienol) and brassicasterol were isolated from spinach leaves and seed of *Brassica rapa* (rapeseed), respectively. Neutral lipids from these two plant sources were fractionated and saponified as described above in procedures i-iii. The dried nonsaponifiables were dissolved in ethanol and the 3β-hydroxy sterols precipitated with digitonin (13) (Sigma). The washed digitonides were refluxed in acetic anhydride to form steryl acetates (13), which recrystallized upon cooling. A second recrystallization was then performed from hot acetone/ethanol (1:1, v/v).

Without further purification, the steryl acetates from spinach were dissolved in benzene and hydrolyzed with 0.5 N sodium methoxide in dry methanol (18) to yield free sterols (≅70% spinasterol). As brassicasterol composed only ≅10% of the phytosterols from rapeseed oil, further purification was required. This was accomplished through argentation column chromatography (14) (four repetitions) followed by argentation TLC (22). The brassicasteryl acetate obtained by these procedures was 94% pure as determined by GLC and was hydrolyzed to the free sterol as described above for the spinach steryl acetates.

RESULTS

Growth-promoting phytosterols. Eleven 3β-hydroxy sterols, two steryl esters, two steryl acetates, one keto steroid and one pentacyclic triterpenoid alcohol were tested for the ability to support growth of *P. tetraurelia* in monoxenic culture when supplied at 5 μg/ml (Table 2). Six C_{29} phytosterols (stigmasterol, sitosterol, spinasterol, stigmastanol, Δ^{22}-stigmastenol and fucosterol), one C_{28} phytosterol (brassicasterol) and the C_{29} 3-keto steroid $\Delta^{4,22}$-stigmastadienone were found to support growth, enabling ≥5 cell divisions with complete clearing of the *E. coli* suspension. All of these steroids, with the exception of fucosterol, which was not tested, were found to promote growth in axenic culture as well (Table 2). Stigmastanol and Δ^{22}-stigmastenol, both C_{29} with a saturated ring system, were shown for the first time to be growth-promoting sterols. As reported previously (3),

TABLE 2

Uptake and Growth Support of Steroids in Monoxenic (M) and Axenic (A) Cultures of *P. tetraurelia*

Steroid/triterpenoid	Carbon no.	Unsaturation	R at C-24	Uptake		Growth	
				M	A	M	A
Stigmasterol	C_{29}	$\Delta^{5,22}$	α-C_2H_5	+	+	+	+
Sitosterol	C_{29}	Δ^{5}	α-C_2H_5	+	+	+	+
Δ22-Stigmastenol	C_{29}	Δ^{22}	α-C_2H_5	+	+	+	+
Stigmastanol	C_{29}	Δ^{0}	α-C_2H_5	+	+	+	+
Spinasterol	C_{29}	Δ^{7}	α-C_2H_5	+	+	+	+
Fucosterol	C_{29}	$\Delta^{5,24(28)}$	$-C_2H_4$	+	N.D.[a]	+	N.D.
Brassicasterol	C_{28}	$\Delta^{5,22}$	β-CH_3	+	+	+	+
Campestanol	C_{28}	Δ^{0}	α-CH_3	+	+	N.D.	N.D.
Campesterol	C_{28}	Δ^{5}	α-CH_3	+	+	0	0
Ergosterol	C_{28}	$\Delta^{5,7,22}$	β-CH_3	+	+	0	0
Cholesterol	C_{27}	Δ^{5}	−H	+	+	0	0
Lanosterol	C_{30}[b]	$\Delta^{5,24}$	−H	+	N.D.	0	N.D.
Stigmasteryl acetate	C_{29}[c]	$\Delta^{5,22}$	α-C_2H_5	+	+	0	+
Ergosteryl acetate	C_{28}[c]	$\Delta^{5,7,22}$	β-CH_3	+	N.D.	0	N.D.
Cholesteryl myristate	C_{27}[c]	Δ^{5}	−H	+	N.D.	0	N.D.
Cholesteryl oleate	C_{27}[c]	Δ^{5}	−H	+	N.D.	0	N.D.
Stigmastadienone	C_{29}	$\Delta^{4,22}$	α-C_2H_5	+	+	+	+
Tetrahymanol	C_{30}[d]	Δ^{0}	—	+	N.D.	0	N.D.

Uptake of the listed steroids in monoxenic and axenic cultures was determined by coretention with standards on gas liquid chromatography (GLC) following extraction and column fractionation of lipids from washed whole cells (see Materials and Methods). In the case of cholesteryl ester supplementation, the steryl ester column fraction was subjected to alkaline methanolysis followed by GLC analysis of the free sterols yielded by this procedure. The criterion set for growth support (+) by a given steroid in both monoxenic and axenic cultures was completion of ≥5 cell divisions 72-96 hr postinoculation. For monoxenic cultures, this also entailed complete clearing of the *E. coli* suspension. (Note the exception to the criterion set for growth support by campesterol and ergosterol in monoxenic cultures cited in Results.)

[a]N.D., No data.

[b]4,4′,14-Trimethyl.

[c]Free sterol C#.

[d]4,4′-Dimethylpentacyclic triterpenoid alcohol.

stigmasteryl acetate supported growth of *Paramecium* in axenic culture. However, in monoxenic culture this compound repeatedly failed to promote growth.

Uptake and metabolism of non-growth-promoting steroids and triterpenoids. P. tetraurelia in monoxenic cultures supplemented with 1 μg/ml stigmasterol was tested for the ability to take up and metabolize the sterols, sterol derivatives and the pentacyclic triterpenoid tetrahymanol (added at 5 μg/ml), which were found not to support growth (Table 2). These data were not tabulated, but are summarized in the following two paragraphs.

Each of the free sterols, the two steryl acetates and tetrahymanol were taken up readily; they comprised 50–90% of the total steroid/triterpenoid pool. Cholesteryl esters were taken up freely, and cholesterol plus 7-dehydrocholesterol comprised >50% of the free sterol fraction. Also, at the time of harvest ≅30% of the ester in the medium had been hydrolyzed. As cholesteryl esters were not hydrolyzed during autoclaving or during incubation with *E. coli* alone (see Materials and Methods), we assume that the deesterification was performed by *Paramecium*. Ergosteryl acetate was not deacylated, and stigmasteryl acetate was not dehydrogenated to the 7-dehydro derivative.

Non-growth-promoting sterols included cholesterol (C_{27}, Δ^5), lanosterol (C_{30}, $\Delta^{8,24}$, 4,4′,14-trimethyl), campesterol (C_{28}, Δ^5) and ergosterol (C_{28}, $\Delta^{5,7,22}$). The latter two C_{28} sterols initially appeared to support growth in monoxenic culture, but failed to do so in subsequent subcultures. Also, in axenic culture these two sterols clearly failed to promote growth. Campestanol (C_{28}, Δ^0), a 30% contaminant of the Sigma stigmastanol preparation, is presumed not to support growth, based on the data for related C_{28} sterols (Table 2 and ref. 3). Cholesterol, campesterol and campestanol were dehydrogenated to yield the $\Delta^{5,7}$-diene derivatives. Lanosterol and ergosterol were not further dehydrogenated.

Metabolism of growth-promoting phytosterols in monoxenic cultures. Monoxenic cultures of *P. tetraurelia* supplemented with the phytosterols listed in Table 3 were harvested at early stationary phase and analyzed for dehydrogenation and esterification of the supplemental sterols. The Δ^5 sterols stigmasterol, sitosterol, fucosterol and brassicasterol were dehydrogenated at the 7,8 position of the B ring, yielding 7-dehydrostigmasterol, 7-dehydrositosterol, 7-dehydrofucosterol and ergosterol, respectively. Spinasterol, the Δ^7 analog of stigmasterol, was dehydrogenated at the 5,6 position, yielding 7-dehydrostigmasterol.

TABLE 3

Metabolism of Growth-Promoting Phytosterols: Monoxenic Cultures

Sterol supplement	Conc. (μg/ml)	Major metabolites—free sterol	%[a]	Esterified sterols	%	FS:SE[b]
Stigmasterol	5.0	7-Dehydrostigmasterol	24	7-Dehydrostigmasterol	85	20:1
		Unknown A	5	Stigmasterol	15	
Δ^{22}-Stigmastenol	5.0	7-Dehydrostigmasterol	24	Δ^{22}-Stigmastenol	75	≥200:1
		Unknown A	10	Cholesterol?	15	
Stigmastanol	5.0	7-Dehydrostigmasterol	17	Campestanol	81	≥100:1
+ Campestanol	2.5	7-Dehydrocampesterol	9	Stigmastanol	15	
		Unknown B	3			
Fucosterol	5.0	7-Dehydrofucosterol	24	No data		No data
		Unknown B	5			
Stigmastadienone ($\Delta^{4,22}$-3-one)	5.0	7-Dehydrostigmasterol	38	7-Dehydrostigmasterol	80	10:1
		Unknown A	15	Δ^{22}-Stigmasterol	20	
		Δ^{22}-Stigmastenol	8			
Sitosterol	5.0	7-Dehydrositosterol	26	Stigmasterol	57	≥200:1
		Unknown B	10	Sitosterol	28	
				Cholesterol?	12	
Spinasterol (70%)	2.0	7-Dehydrostigmasterol	73	None detected		>200:1
		Unknown A	8			
Brassicasterol	4.0	Ergosterol	38	None detected		>200:1

Monoxenic cultures were supplemented at the indicated concentrations with phytosterols shown to support growth in the previous series of experiments (Table 2). Cultures were harvested at early stationary phase (70–80 hr growth; nearly cleared of *E. coli*, 0.9–1.2 × 10^4 cells/ml). Lipids were extracted from washed whole cells and fractionated by silicic acid column chromatography prior to analysis of the FS and SE fractions. "Major metabolites" column presents the percentage of the total FS fraction represented by dehydrogenated and de-ethylated derivatives of the phytosterol(s) supplemented to the culture medium. "Esterified sterols" column presents the percentage of the total SE fraction represented by fatty acyl esters of both metabolized and unmetabolized phytosterols. Percentages of the various sterols included in the FS and SE fractions were determined by gas liquid chromatography (GLC) aided by mass spectrometry. SE were cleaved by alkaline methanolysis prior to GLC analysis of the sterol composition of this fraction. The total sterol mass in both the FS and SE fractions was quantified by integration of GLC peak areas followed by comparison of these with the peak area of a stigmasterol standard solution of known concentration (1.0 mg/ml in 2,2,4-trimethylpentane). The FS to SE mass ratio was then calculated from the values obtained by this method. (See Materials and Methods for details of culturing and lipid analysis.)

[a]Percentage of the total free sterol fraction (i.e., both metabolized and unmetabolized sterols).

[b]FS:SE, free sterol to steryl ester mass ratio.

The two C_{29} sterols with a saturated ring system, Δ^{22}-stigmastenol and stigmastanol, were dehydrogenated at both the 5,6 and 7,8 positions of the B ring, yielding 7-dehydrostigmasterol and 7-dehydrositosterol, respectively. The 3-keto steroid $\Delta^{4,22}$-stigmastadienone was reduced at the 3 position and hydrogenated at Δ^4 to yield Δ^{22}-stigmastenol. The identity of this metabolite was established by GLC retention time and by the very close match of its mass spectrum with that of Δ^{22}-stigmasten-3β-ol isolated from *Dictyostelium*. As indicated above, stigmastenol was further desaturated at both the 5,6 and 7,8 positions, yielding 7-dehydrostigmasterol.

In monoxenic cultures supplemented with either stigmasterol, Δ^{22}-stigmastenol, fucosterol, stigmastanol plus campestanol or sitosterol, 5,7-diene sterols composed $\cong$25% of the free sterol pool. The percentage of $\Delta^{5,7}$ sterols in the free sterol fraction was somewhat higher ($\cong$38%) in cultures supplemented with either brassicasterol or stigmastadienone. In the latter case, the ketone comprised $\cong$40% of the total steroids, excluding SE. Cultures supplemented with spinasterol had an exceptionally high percentage (>80%) of 5,7-diene sterols, principally 7-dehydrostigmasterol, in the free sterol pool.

In addition to the 5,7-diene sterols produced via dehydrogenation of the Δ^0, Δ^5 or Δ^7 phytosterols supplied in the medium, the free sterol pool of cultures supplemented with any of the C_{29} phytosterols included one of two unknowns (designated as unknowns A and B) in the range of 5–15% of the total. Results of argentation column chromatographic fractionation plus GLC and mass spectral analysis of these unknowns strongly suggest their identification as $\Delta^{5,7,22}$-cholestatrien-3β-ol (unknown A) and 7-dehydrocholesterol (unknown B). These sterols probably are formed via deethylation at C-24 of 7-dehydrostigmasterol and 7-dehydrositosterol, respectively.

The steryl ester fraction from stationary phase monoxenic cultures of *P. tetraurelia* in general comprised a small percentage (between 0.5 and 10%) of the total cellular sterols. The SE pool was largest in cultures supplemented with either stigmasterol or stigmastadienone, where 7-dehydrostigmasterol constituted 80–85% of the esterified sterol and stigmasterol or Δ^{22}-stigmastenol the remaining 15–20%. In cultures supplemented with Δ^{22}-stigmastenol, stigmastanol plus campestanol or sitosterol, the SE pool was 10–20 times smaller. The SE fractions from these cultures contained virtually no 5,7-diene sterols, in contrast to SE of cells grown on stigmasterol or stigmastadienone. In cells grown on brassicasterol (4 μg/ml) or spinasterol (2 μg/ml), SE were negligible. Monoxenic cultures supplemented with fucosterol were not analyzed for SE content. The apparent presence of cholesterol (based solely on GLC retention time) in the SE fraction of cultures supplemented with either Δ^{22}-stigmastenol or sitosterol is puzzling, but could be the result of contamination of the supplemental sterols.

Effect of exogenous sterol supplement on the free sterol composition of whole cells and cilia from monoxenic cultures. Table 4 presents the distribution of both growth-promoting and non-growth-promoting sterols (and their 7-dehydro derivatives) in whole cells and cilia from monoxenic cultures. When one of the three phytosterols stigmasterol, fucosterol or campesterol was added alone to the medium, the ratio of the added Δ^5-sterol to its 7-dehydro derivative was $\cong$3:1 in whole cells compared to $\cong$1:3 in cilia. Thus, as indicated in a previous study (5),

TABLE 4

Effect of Exogenous Sterol Supplement on the Free Sterol Composition of Whole Cells and Cilia: Monoxenic Cultures

Sterol supplement	Conc. (μg/ml)	Major free sterols of whole cells	%[a]	Major free sterols of cilia	%[a]
Stigmasterol	5.0	Stigmasterol	74	Stigmasterol	22
		7-Dehydrostigmasterol	19	7-Dehydrostigmasterol	72
Fucosterol	5.0	Fucosterol	71	Fucosterol	17
		7-Dehydrofucosterol	24	7-Dehydrofucosterol	63
Campesterol[b]	2.5	Campesterol	72	Campesterol	20
		7-Dehydrocampesterol	27	7-Dehydrocampesterol	74
Cholesterol	1.0	Cholesterol	38	Cholesterol	4
+		7-Dehydrocholesterol	29	7-Dehydrocholesterol	73
stigmasterol	0.25	Stigmasterol	23	Stigmasterol	2
		7-Dehydrostigmasterol	10	7-Dehydrostigmasterol	22
Ergosterol	5.0	Ergosterol	78	Ergosterol	75
+		Stigmasterol	10	Stigmasterol	5
stigmasterol	0.25	7-Dehydrostigmasterol	5	7-Dehydrostigmasterol	8
Tetrahymanol	5.0	Tetrahymanol	77	Tetrahymanol	43
+		Stigmasterol	19	Stigmasterol	5
stigmasterol	0.5	7-Dehydrostigmasterol	4	7-Dehydrostigmasterol	52

Monoxenic cultures were supplemented with either a high concentration of a growth-promoting phytosterol alone, or a high concentration of a non-growth-promoting sterol plus a low concentration of stigmasterol. Cultures were harvested at early stationary phase, and the washed cells were deciliated by a calcium shock procedure modified from that of Adoutte et al. (9). Cell bodies and cilia were analyzed for their free sterol composition by gas liquid chromatography.

[a]Data are intended to show relative proportions of the major sterols. Minor sterols comprise remaining percentages.

[b]Campesterol alone does not promote growth (Table 2), but did support growth well on the first subculture with stigmasterol-grown cells.

$\Delta^{5,7}$-sterols appear to be preferentially introduced into the ciliary membrane.

In experiments in which a "high" concentration of a non-growth-promoting sterol/triterpenoid was included in the medium (cholesterol, ergosterol or tetrahymanol), a low concentration of stigmasterol was also included as the growth-promoting sterol. Under these conditions, the non-growth-promoting compound constituted ⩾70% of the free "sterol" pool of whole cells, and in the cases of ergosterol and cholesterol plus 7-dehydrocholesterol, ⩾75% of the ciliary sterols. In contrast, tetrahymanol replaced less than half of the ciliary sterols (43%), despite a 10-fold excess over stigmasterol in the medium. This result was not surprising in light of the structural differences of tetrahymanol from the C_{27}, C_{28} and C_{29} sterols and the apparent preferential incorporation of $\Delta^{5,7}$-sterols into the ciliary membrane.

As we have found previously (5), only free sterols, and not steryl esters, are found in cilia of both monoxenic cells (Table 4) and axenic cells (data not shown) of *P. tetraurelia* 51S.

Metabolism of growth-promoting phytosterols in axenic cultures. Axenic cultures of *P. tetraurelia* with or without cholesterol (see Materials and Methods) supplemented with the phytosterols listed in Table 5 were harvested at mid-log phase and analyzed for dehydrogenation and esterification of the supplemental sterols. As expected, the level of cholesterol and its 7-dehydro derivative was higher in the free sterol pool of cultures grown on axenic medium containing the higher concentration of cholesterol. Regardless of the cholesterol level or the phytosterol supplement, 5,7-dienes constituted ⩾70% of the free sterols in axenic cultures. This was generally 2–3 times higher than the percentage of $\Delta^{5,7}$-sterols in comparable monoxenic cultures. The level of $\Delta^{5,7}$-sterols was exceptionally high (⩾95%) in axenic cultures supplemented with spinasterol. This was also observed in monoxenic cultures. For each of the eight growth-promoting steroids, the major metabolites were the same in axenic and monoxenic cultures. Thus, Δ^{5}-sterols were 7-dehydrogenated, Δ^{7}-sterol was 5-dehydrogenated, Δ^{0}- or Δ^{22}-sterols were 5,7-dehydrogenated, $\Delta^{4,22}$-stigmastadien-3-one was converted to Δ^{22}-stigmastenol and in turn to 7-dehydrostigmasterol, and there was evidence of deethylation of 7-dehydrostigmasterol to yield $\Delta^{5,7,22}$-cholestatrien-3β-ol.

The SE pool of axenic cultures was far larger than that of equivalent monoxenic cultures, regardless of the phytosterol supplement or cholesterol level. The percentage of total sterols (free plus esterified) in the SE fraction varied with the growth-promoting sterol supplement and the cholesterol level. Cholesterol appeared to promote esterification with some supplements (brassicasterol and spinasterol), but had the opposite effect with others (Δ^{22}-stigmastenol, stigmastanol plus campestanol, and sitosterol). Only in cultures supplemented with stigmasterol was the sterol composition of the SE fraction unaffected by the presence of cholesterol in the axenic medium; 7-dehydrostigmasterol and stigmasterol in a ratio of ⩾2–3:1 comprised >90% of the esterified sterol. For all other phytosterol supplements, axenic cultures grown on high cholesterol medium included this sterol and its 7-dehydro derivative as major constituents of the SE pool, ranging from 44–95% of the esterified sterol. The sterol composition of SE from cultures grown on low cholesterol axenic medium generally resembled the composition of the free sterol pool when the supplements were stigmasterol, Δ^{22}-stigmastenol, sitosterol or stigmastadienone. With either stigmastanol plus campestanol or spinasterol supplementation, the SE fraction of low cholesterol axenic cultures was greatly enriched in unmetabolized (i.e., not dehydrogenated) sterols. This correlated with a high percentage (≅30%) of phytosterol contaminants (mainly campestanol or Δ^{7}-stigmastenol) in these two sterol supplements. In low cholesterol axenic cultures supplemented with 2 μg/ml brassicasterol, cholesterol was the major esterified sterol in the relatively small steryl ester pool.

Fatty acids of steryl esters vs triglycerides from monoxenic and axenic cultures. A comparison was made of the fatty acid profiles of SE vs TG from both monoxenic and axenic cultures with several different phytosterol supplements. In the case of monoxenic cultures (Table 6), the only supplements yielding sufficient SE for a reliable analysis of the fatty acid profile were stigmasterol and stigmastadienone. Analyses of TG fatty acids from monoxenic cultures supplied with Δ^{22}-stigmastenol, stigmastanol plus campestanol, or sitosterol were also performed. The SE and TG fatty acids were compared for axenic cultures supplemented with stigmasterol, sitosterol, spinasterol or brassicasterol. In the case of the latter two phytosterol supplements, analyses were performed for axenic cultures both with and without cholesterol, while for stigmasterol and sitosterol supplements only cultures including cholesterol were analyzed (Table 7).

The major differences in the FAME profiles of SE vs TG from monoxenic cultures (Table 6) were the ratio of the two cyclopropane fatty acids of *E. coli* (9,10-methylene-hexadecanoic acid and 11,12-methyleneoctadecanoic acid) (19), and the proportion of palmitate (16:0) and palmitoleate (16:1). The 17:cyc to 19:cyc ratio was ≅1:5 for SE FAME and ≅1.6:1 for TG FAME in monoxenic cultures supplemented with either stigmasterol or stigmastadienone. Also, the proportion of 16:0 plus 16:1 was twofold higher in triglycerides than in steryl esters. In both SE and TG, *cis*-vaccenate (*cis*-11-octadecenoic acid) was the major fatty acid, ranging from ≅35–48%. The percentage of 18:1(11) in SE vs TG was slightly higher in monoxenic cultures supplemented with stigmasterol and significantly higher in stigmastadienone-supplemented cultures.

As was the case for monoxenic cultures, the percentage of 16:0 was considerably higher (two- to threefold) in TG compared with SE from axenic cultures, regardless of the cholesterol concentration in the medium or the phytosterol supplement (Table 7). Comparable with the data for monoxenic cultures, *cis*-9-octadecenoic acid (oleic acid) was the major fatty acid in both SE and TG of all axenic cultures, ranging from 38 to 57%. For axenic cultures in which cholesterol constituted a large percentage of the esterified sterol, the amount of 18:1(9) was significantly greater in SE vs TG. In contrast, axenic cultures, which included a large percentage of 7-dehydrostigmasterol in the steryl ester pool, had equal levels of 18:1(9) in SE and TG, but also had a significantly greater proportion of polyunsaturated 20-carbon fatty acids in SE, particularly arachidonate (20:4). Thus, there appears to be some

TABLE 5

Metabolism of Growth-Promoting Phytosterols: Axenic Cultures With and Without Cholesterol (≅2 μg/ml)

Sterol supplement	Conc. (μg/ml)	Major metabolites (free sterol)	%[a]	Esterified sterols	%	FS:SE[b]
With cholesterol						
Stigmasterol	5.0	7-Dehydrostigmasterol	67	7-Dehydrostigmasterol	63	25:75
		7-Dehydrocholesterol	17	Stigmasterol	35	
Δ^{22}-Stigmastenol	3.0	7-Dehydrostigmasterol	62	Cholesterol	93	80:20
		7-Dehydrocholesterol	25	Δ^{22}-Stigmastenol	3	
				7-Dehydrostigmasterol	2	
				7-Dehydrocholesterol	2	
Stigmastanol +	3.4	7-Dehydrositosterol	49	Cholesterol	68	40:60
campestanol	1.6	7-Dehydrocampesterol	15	7-Dehydrocholesterol	24	
		7-Dehydrocholesterol	13	Campestanol	5	
Stigmastadienone	5.0	7-Dehydrostigmasterol	54	Cholesterol	52	30:70
($\Delta^{4,22}$-3-one)		7-Dehydrocholesterol	20	7-Dehydrocholesterol	26	
		Δ^{22}-Stigmastenol	10	7-Dehydrostigmasterol	14	
				Δ^{22}-Stigmasterol	7	
Sitosterol	4.0	7-Dehydrositosterol	60	Cholesterol	37	60:40
		7-Dehydrocholesterol	7	7-Dehydrositosterol	37	
				Sitosterol	18	
				7-Dehydrocholesterol	7	
Spinasterol (70%)	2.0	7-Dehydrostigmasterol	64	Cholesterol	40	50:50
		7-Dehydrocholesterol	25	7-Dehydrocholesterol	23	
				Spinasterol + 7-dehydrostigmasterol	15	
				Δ^{7}-Stigmastenol + 7-dehydrositosterol	17	
Brassicasterol	2.0	Ergosterol	76	Cholesterol	66	30:70
				Brassicasterol	20	
				Ergosterol	13	
Without cholesterol						
Stigmasterol	5.0	7-Dehydrostigmasterol	61	7-Dehydrostigmasterol	69	30:70
		7-Dehydrocholesterol	9	Stigmasterol	24	
		Unknown A	7	Unknown A	5	
Δ^{22}-Stigmastenol	5.0	7-Dehydrostigmasterol	66	7-Dehydrostigmasterol	61	40:60
		Unknown A	19	Δ^{22}-Stigmastenol	36	
		7-Dehydrocholesterol	8	Unknown A	3	
Stigmastanol +	3.4	7-Dehydrositosterol	59	Campestanol	41	15:85
campestanol	1.6	7-Dehydrocampesterol	18	Stigmastanol	30	
				7-Dehydrositosterol	20	
Stigmastadienone	5.0	7-Dehydrostigmasterol	54	7-Dehydrostigmasterol	71	40:60
($\Delta^{4,22}$-3-one)		7-Dehydrocholesterol	20	Δ^{22}-Stigmastenol	27	
		Δ^{22}-Stigmastenol	10			
Sitosterol	5.0	7-Dehydrositosterol	62	7-Dehydrositosterol	63	30:70
		7-Dehydrocholesterol	10	Sitosterol	11	
				7-Dehydrocholesterol	9	
Spinasterol (70%)	2.0	7-Dehydrostigmasterol	71	7-Dehydrostigmasterol	28	75:25
		7-Dehydrocholesterol	25	Spinasterol	23	
				7-Dehydrositosterol	14	
				Δ^{7}-Stigmastenol	14	
				7-Dehydrocholesterol	9	
				Cholesterol	9	
Brassicasterol	2.0	Ergosterol	66	Cholesterol	59	90:10
				Brassicasterol	29	
				Ergosterol	10	

Cells were grown in Soldo's crude axenic medium supplemented with growth-promoting phytosterols (see Table 2) at the indicated concentrations. One series of cultures contained ≥2 μg/ml cholesterol as a contaminant of the medium (with cholesterol), while in a second series ≥90% of the cholesterol was removed (without cholesterol). Axenic cultures were harvested at mid-log phase (70–80 hr growth; 6–8 × 10^3 cells/ml). Lipids were extracted from the washed cells and fractionated by silicic acid column chromatography. The sterol composition of the free sterol and steryl ester fractions was analyzed by gas liquid chromatography and mass spectrometry. "Major metabolites" presents the percentage of the total free sterol fraction represented by dehydrogenated and deethylated derivatives of the sterol(s) present in the culture medium, while "Esterified sterol" includes fatty acyl esters of both metabolized and unmetabolized sterols. The ratio of free sterols to steryl esters was derived as described in the legend to Table 3.

[a]Percentage of the total free sterol fraction (i.e., both metabolized and unmetabolized sterols).

[b]Free sterol to steryl ester mass ratio.

specificity in the chain length and degree of unsaturation of fatty acids esterified to different sterols in *Paramecium.*

DISCUSSION

Although a number of free-living ciliated protozoans show an enhancement of growth when supplementary sterols are available (23-27), the genus *Paramecium* appears to be unique in the absolute requirement of phytosterols for growth (1-3,28). Species of the related genus *Tetrahymena* are capable of synthesizing the pentacyclic triterpenoid tetrahymanol, which in the absence of supplements fulfills the structural/functional role of sterols in this organism (29,30). A wide variety of 3β-hydroxy sterols and their acetates or fatty acyl esters can be utilized by species of *Tetrahymena,* and their inclusion in the nutrient medium generally both enhances growth and suppresses synthesis of tetrahymanol (24-27,31-36). In contrast, the phytosterol requirement in *Paramecium* is highly specific with respect to molecular structure (3,4). It is the basis of this structural specificity of growth-promoting phytosterols in *Paramecium* that we seek to determine.

The results presented here and elsewhere (4,5) show clearly that selective uptake is not the explanation for growth support by certain phytosterols in *P. tetraurelia.* A second possible explanation for the sterol specificity is a vital interaction with other membrane components that is critically dependent on molecular structure. For the plasma membrane, at least, this does not appear to be the case. In appropriately supplemented cultures of *Paramecium,* ≥75% of the sterol in the ciliary (plasma) membrane can be replaced by cholesterol and its 7-dehydro derivative, or ergosterol (Table 4), with no obvious deleterious effects on cell growth or morphology (5; unpublished observation). The possibility remains that some other subcellular organelle membrane is restricted to include only growth-promoting phytosterols.

A third hypothetical explanation for the sterol requirement of *Paramecium* is that some metabolic product of growth-promoting phytosterols is essential for growth and/or cell division. Previously, the two well-established metabolic products of 3β-hydroxy sterols in *P. aurelia* were the 7-dehydro derivatives of Δ^5 sterols and fatty acyl esters. Data presented in this study indicate that *Paramecium* possesses a Δ^5 steroid ring desaturase in addition to the Δ^7 desaturase, thus enabling the double desaturation of saturated ring phytosterols (e.g., stigmastanol, campestanol and Δ^{22}-stigmastenol). The activity of these two desaturases results in the efficient production of $\Delta^{5,7}$-sterols via dehydrogenation of both growth-promoting and non-growth-promoting Δ^0, Δ^7 and Δ^5 precursors. Although $\Delta^{5,7}$-sterols are preferentially incorporated into the ciliary membrane of *P. tetraurelia* (32) (Table 4), and therefore are apparently optimal to fulfill the structural requirements of the cell membrane, their production may not be linked with the promotion of growth by phytosterols. A pivotal question in this regard is whether brassicasterol ($\Delta^{5,22}$; C-24 β-CH_3) can truly support growth, as its 7-dehydro derivative, ergosterol, clearly cannot. At present, it seems likely that the apparent promotion of growth by brassicasterol is due to low level contamination with sitosterol, and that only C-24 α-ethyl or ethylidene sterols are able to promote growth (4).

The second established metabolic pathway of 3β-hydroxy sterols in *Paramecium,* fatty acyl esterification, has been suggested by Conner et al. (4) to be linked with the promotion of growth by phytosterols. This conclusion

TABLE 6

Fatty Acyl Methyl Ester (FAME) from Steryl Esters (SE) and Triglycerides (TG): Monoxenic Cultures

	Added sterol (5.0 μg/ml)						
	Stigmasterol		Stigmastadienone		Sitosterol	Stigmastenol	Stigmastanol plus campestanol (2:1)
FAME	SE	TG	SE	TG	TG	TG	TG
16:0	13.9	21.9	15.2	21.8	21.8	20.6	21.4
16:1	1.3	6.8	1.7	9.4	6.1	5.3	5.6
16:2	1.1	0.8	0.8	0.6	0.9	1.1	1.1
17:cyc	4.2	16.8	4.0	15.5	18.2	18.1	17.6
18:0	4.4	1.9	3.2	2.1	1.7	2.1	2.0
18:1(11)	39.6	35.2	47.7	36.8	34.0	32.9	35.0
18:2	4.8	1.4	2.8	1.0	1.9	2.7	2.1
19:cyc	22.8	11.1	18.4	8.7	11.3	14.1	13.0
18:3	2.6	0.5	0.7	0.3	0.6	0.4	0.6
20:2	0.6	0.2	1.6	0.2	0.3	0.2	0.2
20:3	2.3	0.1	1.7	0.2	0.2	0.1	0.1
20:4	1.2	0.1	0.8	0.1	0.2	0.1	0.1

Data indicate the relative percentage of total fatty acids in SE or TG. The fatty acid composition of steryl esters and triglycerides was compared for early stationary phase cells from monoxenic cultures supplemented with either stigmasterol or stigmastadienone at 5.0 μg/ml. These were the only steroid supplements yielding sufficient steryl esters for an accurate analysis of fatty acid composition. For the sake of comparison, the fatty acid composition of triglycerides from monoxenic cultures supplemented with several other phytosterols was also analyzed. The SE and TG fractions were isolated from neutral lipids of whole cells by silicic acid column chromatography. Fatty acids acylated to either sterols or glycerol were transesterified with sodium methoxide to yield FAME for gas liquid chromatographic analysis. (See Materials and Methods for details of lipid analysis.)

TABLE 7

Fatty Acyl Methyl Esters (FAME) from Steryl Esters (SE) and Triglycerides (TG): Axenic Cultures With or Without Cholesterol

	With cholesterol											Without cholesterol			
	Stigmasterol (5.0 μg/ml)		Sitosterol (4.0 μg/ml)		Stigmastadienone (5.0 μg/ml)	Stigmastenol (3.0 μg/ml)	Stigmastanol plus campestanol (2:1) (5.0 μg/ml)	Spinasterol (2.0 μg/ml)		Brassicasterol (2.0 μg/ml)		Spinasterol (2.0 μg/ml)		Brassicasterol (2.0 μg/ml)	
FAME	SE	TG	SE	TG	TG	TG	TG	SE	TG	SE	TG	SE	TG	SE	TG
16:0	10.3	22.0	13.5	24.5	27.4	25.1	27.9	7.1	27.4	8.6	27.6	8.5	22.7	9.3	22.8
16:1	4.1	2.6	8.0	4.0	4.3	4.8	4.4	1.7	3.6	1.3	3.6	1.5	3.2	1.4	3.4
16:2	6.9	0.8	0.8	0.9	1.0	1.0	1.7	0.8	0.8	0.8	0.8	0.5	0.6	0.2	0.6
18:0	5.5	9.4	7.2	7.1	7.5	7.1	7.8	6.7	7.4	8.9	7.1	6.3	8.2	12.0	7.5
18:1(9)	40.0	38.0	56.0	43.7	42.1	43.6	42.7	51.0	41.8	55.8	42.4	42.8	44.2	57.3	44.6
18:2	6.3	9.0	2.7	7.5	5.4	7.1	4.8	3.5	5.4	7.4	5.6	3.8	5.2	2.9	5.3
18:3	4.4	5.9	4.7	3.1	5.2	3.3	4.0	8.3	5.3	9.5	5.6	10.6	6.3	9.7	6.6
20:2	2.8	1.5	0.4	1.1	0.4	1.1	0.3	1.4	0.7	0.8	0.4	0.8	0.7	0.7	0.3
20:3	3.5	2.2	0.4	0.3	0.3	0.4	0.3	2.1	0.5	1.4	0.5	4.9	0.7	1.6	0.8
20:4	12.0	6.0	1.1	0.7	1.6	0.9	1.6	9.3	2.5	3.8	2.5	16.5	2.5	3.3	3.2
20:5	1.5	1.6	2.1	3.4	0.7	3.3	0.6	2.2	0.9	1.0	1.0	1.6	1.5	0.6	0.8

Data indicate the relative percentage of total fatty acids in SE or TG. The fatty acid composition of SE and TG was compared for log phase cells from axenic cultures supplemented with different growth-promoting phytosterols at the indicated concentrations. In the first series of experiments the axenic medium contained ⩾2 μg/ml cholesterol as a contaminant (with cholesterol), while in the second series ⩾90% of the cholesterol was removed from one pair of cultures supplemented with either brassicasterol or spinasterol (without cholesterol). The SE and TG fractions were isolated from neutral lipids of whole cells by silicic acid column chromatography. Fatty acids acylated to either sterols or glycerol were transesterified with sodium methoxide to yield FAME for gas liquid chromatographic analysis. (See Materials and Methods for details of culturing and lipid analysis.)

was based on the results of a study comparing the metabolism of stigmasterol and cholesterol in *P. aurelia*. Growth-promoting stigmasterol and 7-dehydrostigmasterol were esterified, while non-growth-promoting cholesterol and 7-dehydrocholesterol were not. We have confirmed this result for axenic cultures of *P. tetraurelia*, but have also shown that in cultures supplied with cholesterol and a growth-promoting phytosterol other than stigmasterol, fatty acyl esters of cholesterol and 7-dehydrocholesterol can compose >90% of the SE pool. Thus, selective esterification of only growth-promoting phytosterols does not occur. Furthermore, the fatty acid profiles of cholesteryl and stigmasteryl esters from axenic cells were similar, both predominated by the essential fatty acid 18:1(9). Although it appears unlikely at present, it is still possible that fatty acyl esters of growth-promoting phytosterols perform some specialized, vital role in the fatty acid metabolism of *Paramecium*. It seems more probable, based on the data presented here, that steryl esters in *Paramecium* may serve the threefold purpose of storing growth-promoting sterols when they are abundant in the medium, storing essential or useful fatty acids in a nontoxic form and sequestering non-growth-promoting sterols when they are abundant relative to growth-promoting sterols. The last of these functions has been described in the yeast *Saccharomyces cerevisiae* (37,38). The detection of SE in the ciliary membrane of *P. tetraurelia* has recently been reported by Kaneshiro et al. (39). We were unable to confirm this, finding only free sterols as we reported previously (5).

In addition to fatty acyl esterification and 5,6 and/or 7,8 dehydrogenation of 3β-hydroxy sterols, two other facets of sterol metabolism in *Paramecium* were indicated in the course of this work. Data supporting the conclusion that 7-dehydrostigmasterol and 7-dehydrositosterol can be deethylated by *P. tetraurelia* to yield $\Delta^{5,7,22}$-cholestatriene and 7-dehydrocholesterol, respectively, were obtained by GLC, ultraviolet and mass spectrometric analyses (data not shown). Deethylation of phytosterols at C-24 has been demonstrated previously in *Tetrahymena* (40,41), which preferentially incorporates $\Delta^{5,7,22}$-cholestatriene into the ciliary membrane (42). However, the role of this metabolic pathway in *Paramecium* is uncertain, as it results in the conversion of growth-promoting precursors to non-growth-promoting products (4). The anomalous growth support of *P. tetraurelia* by $\Delta^{4,22}$-stigmastadien-3-one has been explained with the demonstrated conversion of this 3-keto steroid to Δ^{22}-stigmastenol, which necessarily entails the reduction of the 3-keto to 3β-hydroxy group and the saturation of the 4,5 double bond. Similar metabolism of a Δ^{4},3-keto steroid has been demonstrated in *Tetrahymena furgasoni* (43).

In summary, the basis of the growth requirement for specific phytosterols in *Paramecium* does not appear to be related to the structural/functional role of sterols in cell membranes. Nor is there any evidence to suggest a link between the major metabolites of sterols in *Paramecium* (i.e., fatty acyl esters and dehydrogenated or deethylated derivatives) and the ability of their precursors to support growth. Recent experiments in this laboratory have shown that cholesterol can spare the requirement for stigmasterol in *P. tetraurelia* if one supplies the latter at a subsupportive concentration (manuscript to be submitted) (Table 4). When cholesterol is provided

at 1.0 μg/ml, the required threshold level of stigmasterol is ≅0.015-0.020 μg/ml in both axenic and monoxenic cultures (manuscript to be submitted). In cells grown to stationary phase with this ratio of cholesterol to stigmasterol, there is virtually no stigmasterol or 7-dehydrostigmasterol in the ciliary membrane. Thus, the "bulk" sterol requirement for membrane biosynthesis in *Paramecium* can be satisfied by the non-growth-promoting sterol cholesterol and its 7-dehydro derivative. Similar results have recently been reported in *Saccharomyces cerevisiae* for both sterol-deficient mutants (44) and anaerobic wild-type cultures treated with an inhibitor of sterol biosynthesis (45,46). An absolute requirement for traces of a Δ^5; C-24, β-methyl; 3β-hydroxy sterol has been demonstrated for this yeast, although a wide variety of sterols can fulfill the "bulk" membrane requirement (44-46). Finally, Guyer and Bloch (27) have recently found that ergosterol can stimulate growth of *Tetrahymena setosa* at a concentration well below that required to inhibit synthesis of tetrahymanol. They conclude that the trace amounts of sterol needed by *T. setosa* do not serve to replace tetrahymanol in membranes, but function in some other manner, perhaps metabolic.

In light of these data, it appears that in *Saccharomyces*, *Paramecium* and *Tetrahymena setosa* there are at least two roles for cellular sterols. The first of these is the structural/functional role in cell membranes, which is quite nonspecific. The second, perhaps "hormonal," role is much more specific with regard to molecular structure and requires a relatively small amount. It remains to be determined exactly what function this minor sterol component serves and whether it is the growth-promoting sterol per se, or some minor metabolite as yet undetected, that carries out this function.

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Electron Microscopic Cytochemistry of Cyclopropenoids in Cottonseed (*Gossypium hirsutum* L.) Tissues

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An electron microscopic cytochemical procedure for locating cyclopropenoids (CYP) in oilseeds is proposed. The procedure is based on the fact that metallic silver is produced when $AgNO_3$ reacts with CYP. Since so many other compounds react with $AgNO_3$, we treated specimens that do and do not contain CYP with $AgNO_3$ and looked for electron-dense stains that were unique to CYP(+) seeds. Virtually everything in all seed specimens, regardless of the presence or absence of CYP, was stained. However, cottonseeds contained two additional staining constituents not found in controls: (i) certain large, intracellular droplets, and (ii) the cuticles, both occurring exclusively in the axial organs. Extraction of the specimens with ether abolished the stainability of the droplets but did not affect cuticles. Arguments for the validity of the test are presented.
Lipids 22, 397-400 (1987).

Cottonseeds contain 30–35% oil, 0.6–1.1% of which is cyclopropenoid fatty acids (1). This small quantity of cyclopropenoid (CYP), however, can cause highly deleterious and undesirable results if ingested by animals. For instance, lard from pigs becomes hard (2), butter from cows becomes sticky and its melting point is raised higher than normal (3) and whites of chicken eggs turn pink in storage (4). CYP also causes elevated plasma and liver cholesterol levels in laboratory animals and a higher incidence of aortic atherosclerosis than normal (5). It causes growth to be stunted when fed in moderate amounts and death when fed in higher amounts (6). CYP acts as a synergist for the carcinogen aflatoxin (7) and has proved to be a carcinogen itself (8). Therefore, although the level of CYP in commercial cottonseed oil is not toxic to humans, and there are many methods of destroying or removing it (9), interest in all aspects of this compound continues. One of the things cottonseed processors have wanted to know is where in the seed CYP is stored.

Fisher and Cherry (10) conducted a histochemical study of cottonseed to find where CYP are stored. They excised various parts of the seeds, extracted the parts with ether and analyzed the ether extracts by gas liquid chromatography. They found the bulk of the ether-extractable cyclopropenoid fatty acids of cottonseed in the axial organs and very little in the cotyledons. More recently, Wood (11) stated that cyclopropenoid fatty acids of cottonseed are located almost exclusively, *if not* totally, in the hypocotyls.

Our task was to find the cytological location of CYP. The procedure that we devised was based on the fact that $AgNO_3$ reacts with CYP to produce metallic silver. Our strategy was to stain both CYP(+) and CYP(−) seeds with $AgNO_3$, then to search for electron dense deposits in CYP(+) seeds that were not in CYP(−) control seeds. Two $AgNO_3$-staining sites were found in cottonseed (both in the axial organs) that were absent from control seeds. They were (i) certain large intracellular droplets and (ii) the cuticle of the epidermal cells. The droplets did not stain after specimens were extracted with ether, but cuticle stainability persisted. We do not yet know the chemistry of the cuticular material but conclude that $AgNO_3$-reactive lipids are stored in the droplets.

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MATERIALS AND METHODS

Cottonseeds were soaked in 2% glutaraldehyde/0.05 M cacodylate buffer, pH 7.0, until soft and pliable; axial organs and cotyledon tissues were excised. The excised specimens were fixed in fresh fixative overnight and serially dehydrated in aqueous ethanols to the 80% ethanol level. Two sets of controls were used: seeds that do not have CYP (e.g., peanut and squash) and cottonseed tissue that was extracted with ether.

Artificial "spherosomes" were prepared as before (12). Briefly, vegetable oils and phospholipids were emulsified in water with a Branson sonicator. A 2% solution of agar was boiled, cooled to 45–40 C and mixed with an equal volume of emulsion (warmed to 40–45 C), then centrifuged. The cream layers on the tops of the centrifuge tubes, immobilized in agar, were cut into specimen-sized pieces and dehydrated serially to 80% alcohol. *Sterculia foetida* oil (13) was used to make CYP-containing spherosomes and peanut and soy oils were used to make control spherosomes.

Spherosomes were harvested from cottonseed axial organs and cotyledon tissues. Cottonseeds were soaked in water briefly (until softened), and respective organs were excised. Each sample was ground in a mortar and pestle with water and fine sand. The homogenates were centrifuged, and the fatty layers were collected for study. (In later studies, the entire operation was conducted at ice temperatures to slow metabolism (14) and to thicken the triglycerides, in hopes of preserving the fat droplets.) Samples for light microscopy were placed on a slide and viewed as is. Specimens to be viewed in an electron microscope were treated in the same manner as with artificial spherosomes above.

CYP cytochemistry was conducted on specimens serially dehydrated to 80% ethanol. The samples were placed into a staining solution of 80% saturated $AgNO_3$ dissolved in 95% ethanol and incubated at 65 C for 4 hr. After incubation, specimens were rinsed thoroughly with 95% ethanol, transferred to acetone and embedded in Spurr's epoxy resin. Thin sections were cut on a Porter-Blum MT-2 ultramicrotome with a diamond knife and viewed in a Philips EM-200 electron microscope. We refrained from using metallic fixatives or stains to avoid confusion with stains imparted by $AgNO_3$. Control tissues were treated in the same manner.

RESULTS AND DISCUSSION

Almost a hundred years ago it was noted that cottonseed oil reacts with $AgNO_3$. This information was used as the

basis for a test to detect cottonseed oil adulteration of olive oil (15). More recently, Kircher (16) showed that brown spots that developed on his lipid chromatograms when sprayed with alcoholic $AgNO_3$ were due to the reaction between $AgNO_3$ and the CYP moiety of sterculic acid. Oxidation of CYP was coupled to the reduction of $AgNO_3$ to form metallic silver. We wondered if this reaction would take place in situ in biological tissues; if so, could $AgNO_3$ serve as an electron microscopic cytochemical stain for CYP?

Because neutral lipids extracted from cottonseed meals contain CYP (1,10,11,17) and the storage site of neutral lipids in cottonseed is in intracellular fat droplets, or spherosomes (18), our first guess was that the CYP fatty acids would be found in spherosomes. In a preliminary feasibility test, artificial spherosomes (emulsion droplets) were stained with alcoholic $AgNO_3$ and examined in an electron microscope. Triglyceride droplets maintained their integrity in a 95% ethanolic stain carrier. No electron-dense stains were observed within or upon peanut or soy oil droplets (which do not have CYP), but myriad of electron-dense particles were seen on the surfaces of *Sterculia foetida* oil droplets (which do have CYP). $AgNO_3$ is hydrophilic and CYP-triglyceride is hydrophobic, so the reaction took place where the two phases meet—at the surface of the droplets.

Cottonseed specimens, which were a light cream color, turned to various shades of brown, depending on the

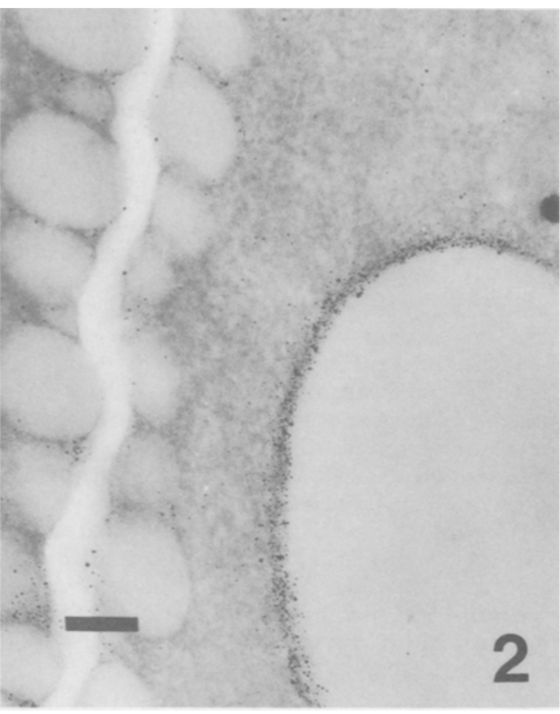

FIG. 2. A portion of Fig. 1 at higher magnification. Notice that the dark fringe around the droplet is actually a mass of fine, electron-dense specks. Although the diameter of the large droplet is only 3 to 10 times that of ordinary spherosomes, the volume is on the order of 27 to 1000 times. Bar indicates 0.5 μ.

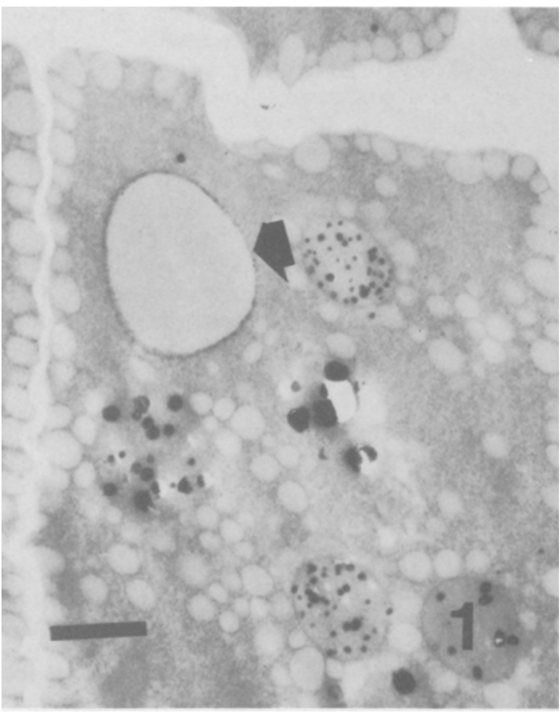

FIG. 1. Low magnification electron micrograph showing a portion of a cottonseed axial organ cell stained with $AgNO_3$. Note the large droplet in the upper left-hand corner of the cell with a dark fringe (large arrow); the large droplets are 3 to 10 times as large as ordinary spherosomes. This type of droplet and the cuticles stained in cottonseeds but not in controls. Another heavily staining site—the globoids in the protein bodies—is also visible. Globoids are not exclusive to cottonseeds. Bar indicates 2 μ.

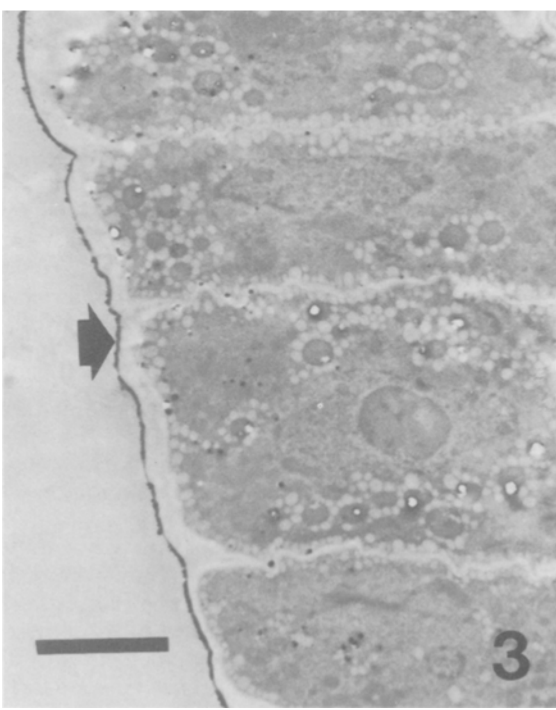

FIG. 3. Low magnification electron micrograph of some epidermal cells on a cottonseed axial organ. Note the dense deposition of silver on the cuticle (large arrow). Organelles are difficult to discern. The nucleolus and chromatin material of the nuclei and the aleurone grains tend to be slightly darker than the groundplasm of the cell. Some aleurone grains have holes at sites formerly occupied by globoids (see Fig. 1). Bar indicates 5 μ.

organ, when immersed in alcoholic $AgNO_3$. Axial organs turned brown quickly (within minutes), with colors ranging from light to very dark brown. The radicular region generally stained darkest; the tips (root caps) often remained much lighter. Interestingly, the outer surfaces of the axial organs often acquired a metallic sheen after $AgNO_3$ treatment. Cotyledons remained a light cream color for a long time, but eventually turned light tan.

Because silver nitrate reacts with most biologically important compounds (19–22), the strategy used in this study was to treat seeds that do and do not contain CYP with $AgNO_3$ and to look in CYP(+) seeds for electron-dense stains that were absent from CYP(−) seeds. Virtually all cytoplasmic elements were stained by alcoholic $AgNO_3$, regardless of the presence or absence of CYP. The cytoplasmic ground substance in all seeds examined was lightly stained with silver (Figs. 1–3); aleurone grains stained slightly darker. Nuclear ground plasma was weakly stained, with the nucleolus and chromatin material staining somewhat stronger. Ordinary spherosomes (in contrast to the large droplets discussed below) were *electron lucent with no* electron-dense stains within or upon the droplet surfaces. Cell walls were essentially unstained. Globoids, a notable exception, were stained very heavily (the chelation product of silver by phytic acid). Membranes were not stained by $AgNO_3$ (as they are with OsO_4 or permanganate); in fact, they often appeared in negative contrast. Consequently, organelles were difficult to discern (Figs. 1 and 3). We tried the $AgNO_3$ test on OsO_4-fixed cottonseed tissue but it did not work satisfactorily. OsO_4 not only caused the unsaturated lipids to turn black and smeary but, more importantly, caused numerous extraneous electron-dense deposits to appear when poststained with $AgNO_3$.

Three $AgNO_3$-staining sites were found in cottonseeds that were not present in control seeds. One site, the pigment gland, has already been eliminated as a site of CYP storage in cottonseeds (23). The stains in the glands were due to the reaction of silver ions with gossypol and/or related polyphenolic compounds (24). The other two sites, both in the axial organs, were (i) certain intracellular droplets and (ii) the cuticles of the epidermal cells. Droplets that stained with $AgNO_3$ had the same electron-density as ordinary spherosomes but were much larger, and tended to be embedded in or associated with cytoplasmic ground substance (Fig. 1). Silver particles that deposited on the surfaces of these large droplets were very small and fine compared to the large and coarse particles seen on the surfaces of *S. foetida* oil droplets. This may have been due to the fact that emulsion droplets have no membranes, whereas spherosomes are bounded by a "half unit membrane" (25), or perhaps because *S. foetida* oil has such a high concentration of CYP (13). Morphologically similar droplets in the axial organs of ether-extracted cottonseeds did not stain with $AgNO_3$, suggesting that a $AgNO_3$-reactive neutral lipid was extracted from the droplets.

Authentic spherosomes extracted from cottonseed axial organs turned yellow-brown when treated with $AgNO_3$, but spherosomes extracted from cottonseed cotyledons remained white. Light microscopic examination of $AgNO_3$-treated axial organ spherosomes showed patches of yellow-brown scattered about in the field, indicating that $AgNO_3$ reactivity was not uniformly distributed among all the fat droplets. We did not find the large droplets (seen in situ) in the isolated spherosome preparations. Instead, the size of the droplets varied widely and electron-dense grains, which were not on all droplets, were large and course (like those seen in the *Sterculia foetida* emulsions). We believe that the large droplets in the axial organs were broken during homogenization. The use of sand or cold temperatures did not seem to help preserve the integrity of the large droplets.

The outer surfaces of the epidermal cell walls (cuticles) were stained heavily with silver. Unlike with the droplets, ether extraction did not abolish the stainability of the cuticular material. We do not yet know the chemical composition of the $AgNO_3$-reactive substance, but because of its insolubility in hexane and apparent insolubility in $CHCl_3$/methanol, it cannot be a neutral lipid and is probably not a phospholipid (26,27). The copious deposits of metallic silver on the surfaces of the axial organs explain the metallic sheen we observed on the surfaces of $AgNO_3$-treated cottonseed axial organs.

Considerable evidence, albeit circumstantial, has been amassed in this study to indicate that CYP-neutral lipids of cottonseeds are located in certain large-sized droplets found in cells of the axial organs. Firstly, vegetable oils that react with $AgNO_3$ to produce a yellow-brown pigment and metallic silver are highly unusual (the only ones we are aware of are CYP lipids). These reactions took place in spherosome fractions extracted from cottonseed axial organs but not in those isolated from CYP(−) control seeds. Certain droplets that were most numerous in the radicular regions of the axial organs (in the same general area of the seeds where Fisher and Cherry [10] found the highest concentration of ether-extractable cyclopropenoid fatty acids) stained with alcoholic $AgNO_3$. Extraction of the tissues with ether abolished the stainability of the droplets. Therefore, we believe that the CYP lipids of cottonseeds are located in the large droplets in seed axial organs.

The results of this study also suggest that a heterogeneous population of lipid droplets exists in cottonseeds (one group that reacts with $AgNO_3$, the other that does not). However, if lipids are synthesized in a central location, e.g., plastids (28), and subsequently are dispersed to fat droplets, it seems reasonable to expect the lipid complement of the various droplets to be rather homogeneous. Because this does not seem to be the case in cottonseeds, CYP lipids must have special synthetic (not necessarily de novo) and storage processes. We wonder whether unusual lipids in other plants are also housed in special droplets. The sequestration of CYP lipids in special droplets answers how cottonseeds are able to synthesize unsaturated fatty acids in spite of the presence of CYP, a fatty acid synthetase inhibitor (29).

To recapitulate, we showed that a silver precipitate (not found in non-CYP seeds) occurred in certain large droplets and cuticles of cottonseed axial organs after alcoholic $AgNO_3$ treatment. The highest incidence of silver-reactive droplets was found in the same location where Fisher and Cherry (10) found the highest concentration of ether-soluble CYP. We found no evidence for such deposits in the cotyledons. Fisher and Cherry (10) found very little CYP in cotyledons, and Wood (11) suggested that there is no CYP in cotyledons. We show that $AgNO_3$ can be a useful tool in cytochemical studies of CYP; however, because reaction with $AgNO_3$ is not the exclusive domain of CYP, caution should be exercised in interpret-

ing such tests. We do not yet know the chemical nature of the $AgNO_3$-reactive substance in the cuticles, but as Halloin (30) suggested, one of the functions of CYP in cottonseeds may be protective, and we cannot rule out the possibility that the $AgNO_3$-reactive substance in the cuticles might be a cyclopropenoid. It will be recalled that high concentrations of CYP have also been reported in the axial organs of the durian seed (31) and in the seeds and roots (but not in the other parts, such as leaves, stems or trunk wood) of *Ceiba pentandra* and *S. foetida (32)*.

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Lipid Peroxidation in Erythrocyte Membranes: Cholesterol Product Analysis in Photosensitized and Xanthine Oxidase–Catalyzed Reactions

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The effects of singlet oxygen- and oxygen radical–induced lipid peroxidation on cell membrane integrity were compared, using the human erythrocyte ghost as a model system. Resealed ghosts underwent lipid peroxidation and lysis (release of trapped glucose-6-P) when irradiated in the presence of uroporphyrin (UP) or when incubated with xanthine (X), xanthine oxidase (XO) and iron. The UP-sensitized process was inhibited by azide but not by phenolic antioxidants, consistent with singlet oxygen (nonradical) involvement. This was confirmed by showing that the predominant photoproduct of membrane cholesterol was the 5α-hydroperoxide. Total hydroperoxide (LOOH) content in UP-photooxidized ghosts increased linearly during the prelytic lag and throughout the period of rapid lysis. Unlike the photoreaction, X/XO/iron-dependent peroxidation and lysis was inhibited by catalase, superoxide dismutase and phenolic antioxidants, indicating O_2^-/H_2O_2 intermediacy and a free radical mechanism. Correspondingly, only radical reaction products of cholesterol were formed, notably the 7α-,7β-hydroperoxide pair. Membrane lysis had a distinct lag as in photooxidation; however, the LOOH profile was more complex, with an initial lag followed by a sharp increase and then slow decline. X/XO/iron-induced lysis commenced when LOOH levels were 2–3 times higher than in photosensitized lysis, suggesting that the pathways of membrane lesion formation are different in the two systems. In low concentrations, ascorbate exacerbated the damaging effects of photoperoxidation, switching the reaction from primarily singled oxygen- to oxygen radical–dependence, as indicated by cholesterol product analysis.
Lipids 22, 401–408 (1987).

Peroxidative degradation of unsaturated phospholipids and cholesterol in biological membranes is known to be deleterious to membrane structure and function. Lipid peroxidation can be triggered by partially reduced oxygen species (superoxide, O_2^-; hydrogen peroxide, H_2O_2; or hydroxyl radical, $OH^\cdot$) generated by oxidase-catalyzed reactions, autoxidation of electron donors (e.g., ascorbate and thiols), ionizing radiation and various other processes. Redox metals often play a role in initiating this type of peroxidation, which is then propagated by alkoxyl or peroxyl radical intermediates (1,2). Singlet molecular oxygen ($^1O_2$), typically generated by energy transfer from excited state dyes or pigments, also oxidizes unsaturated lipids. However, $^1O_2$ attacks directly via the "ene" mechanism, and free radical intermediates are not necessarily involved (3). Although both types of lipid peroxidation have been studied extensively in various model and natural systems, little is known about their relative damaging effects on any particular target membrane. Using resealed erythrocyte ghosts as a test system, we investigated this by tracking degree of lysis as a function of peroxide content in ghosts exposed to xanthine/xanthine oxidase as a source of oxygen radicals and to photoexcited uroporphyrin as a source of $^1O_2$. Definitive evidence for the involvement of these species was obtained by identifying specific oxidation products of cholesterol. Cholesterol product analysis was also used for probing mechanistic changes that occur during photooxidation of ghosts in the presence of ascorbate (4).

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Abbreviations—PBS: phosphate-buffered saline (25 mM sodium phosphate, 125 mM NaCl, pH 7.4); LOOH: lipid hydroperoxide; BHT: butylated hydroxytoluene (2,6-di-t-butyl-4-methylphenol); DTP: 2,6-di-t-butylphenol; 5α-OOH: 3β-hydroxy-5α-cholest-6-ene-4-hydroperoxide; 5α-OH: 5α-cholest-6-ene-3β,5-diol; 7α-OOH: 3β-hydroxycholest-5-en-7α-hydroperoxide; 7β-OOH: 3β-hydroxycholest-5-en-7β-hydroperoxide; 7α-OH: cholest-5-en-3β,7α-diol; 7β-OH: cholest-5-en-3β,7β-diol; G6P: glucose-6-phosphate; TMPD: N,N,N′,N′-tetramethyl-p-phenylenediamine; AH⁻: ascorbate; UP: uroporphyrin I; PP: protoporphyrin IX.

MATERIALS AND METHODS

Materials. Freshly drawn human blood was obtained from the Blood Center of Southeastern Wisconsin (Milwaukee, WI) and used within two weeks. Uroporphyrin I (UP) and protoporphyrin IX (PP) were purchased from Porphyrin Products (Logan, UT); absorption spectral characteristics agreed with those previously reported for these porphyrins (5). Xanthine oxidase (grade III), glucose-6-phosphate (G6P) dehydrogenase (type VII), thymol-free catalase and Cu/Zn-superoxide dismutase were obtained from Sigma Chemical Co. (St. Louis, MO), as were xanthine, NADP, D-G6P, butylated hydroxytoluene (BHT), cholesterol, 3β-hydroxycholest-6-en-7-one (7-ketocholesterol) and egg yolk phosphatidylcholine. N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD), 2,6-di-t-butylphenol (DTP) and 2-thiobarbituric acid (TBA) were from Aldrich Chemical Co. (Milwaukee, WI), and sodium ascorbate (>98%) was from BDH Chemicals (Poole, England). All other chemicals were of highest purity available, and all aqueous solutions were prepared with deionized, glass-distilled water.

Resealed erythrocyte ghosts containing G6P as an encapsulated marker of membrane integrity were prepared as described previously (6). Unsealed ghosts were prepared by conventional hypotonic lysis, followed by washing and concentration in a Millipore-Pellicon tangential flow system (7). Stock membrane suspensions were blanketed with N_2 during storage at 4 C and typically were used within one week. Membrane protein was determined by the method of Lowry et al. (8).

Experimental conditions. Irraditions were carried out in matched, thermostated beakers (45-mm inner diameter) or in vials (20-mm diameter) inserted into 4-place Stirrer Baths (Yellow Springs Instruments, Yellow Springs, OH). Stirred suspensions of well-washed membranes, typically 1.8 × 10^9 ghosts (1 mg protein)/ml in

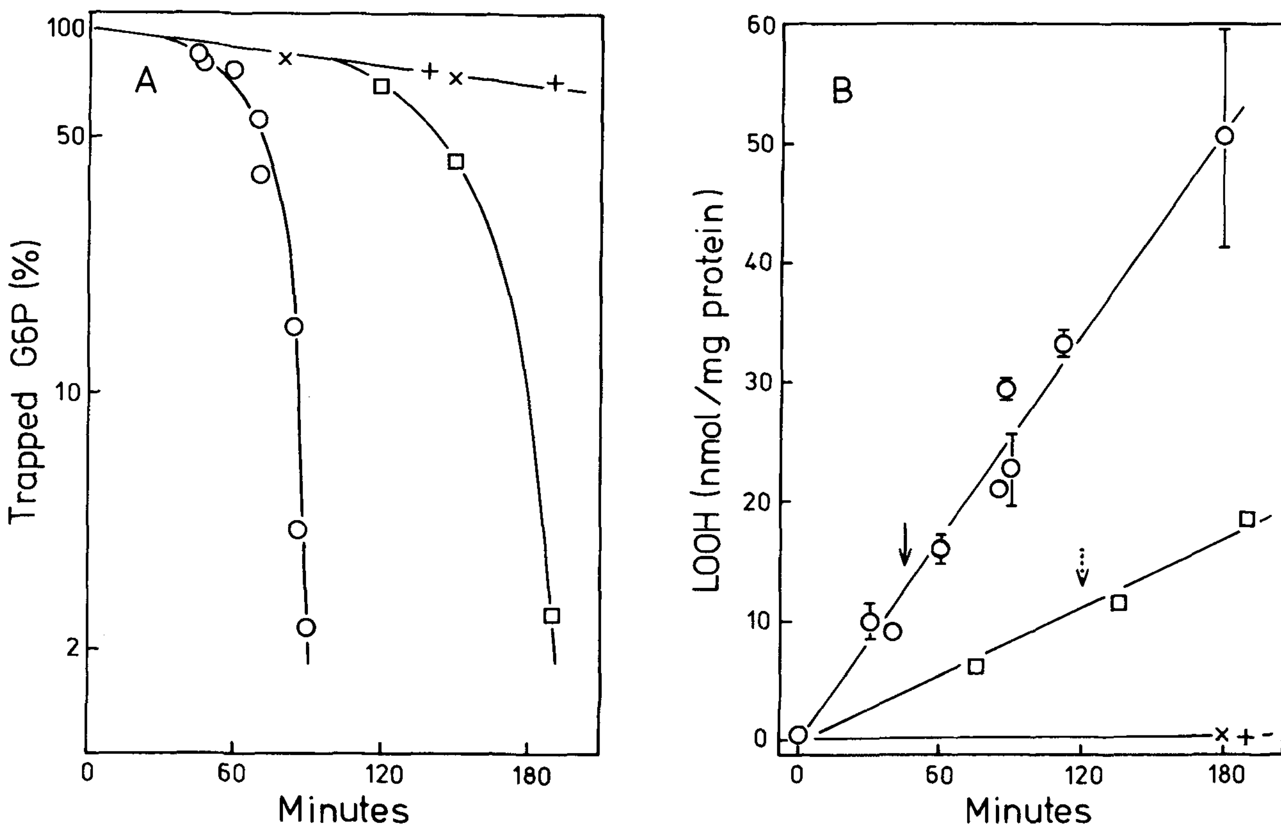


FIG. 1. Uroporphyrin (UP) photosensitized lipid peroxidation and lysis of resealed ghosts. Ghosts (1.0 mg protein/ml in PBS at 37 C) were irradiated continuously in the presence of 2μM UP (□) and 10 μM UP (○). The fluence rate at the suspension surfaces was 20 mW/cm^2. The dark controls contained 2 μM UP (+) and 10 μM UP (×). (A) Membrane lysis, as measured by release of trapped glucose-6-phosphate (G6P); (B) Lipid peroxidation, measured in terms of total hydroperoxide (LOOH) content. Error bars show means ± deviations of values from duplicate determinations. LOOH level at approximate time of incipient lysis is indicated by dotted arrow for 2 μM UP and solid arrow for 10 μM UP.

phosphate buffered saline (PBS; 25 mM sodium phosphate, 125 mM NaCl, pH 7.4), were sensitized with UP or PP and irradiated with broad band blue light as described previously (4). Fluence rate near the suspension surfaces was measured with a Yellow Springs radiometer. Unless indicated otherwise, reactions were run at 37 C. Enzymatic oxidations in the presence of xanthine, xanthine oxidase and iron were carried out as specified earlier (6). Azide was typically included to inactivate any residual catalase in the membranes; it was omitted when catalase was added diagnostically. Samples were removed periodically from reaction mixtures to assess lipid peroxidation (see below) and membrane lysis. Reactions were terminated by adding allopurinol to xanthine oxidase–containing samples and by keeping irradiated samples in the dark. Lysis of resealed ghosts was measured in terms of enhanced G6P efflux (6). Following rapid centrifugation (1–3 min after sampling), the marker was determined by coupling its G6P dehydrogenase–catalyzed oxidation to the reduction of NADP (A_{340}).

Quantitation of lipid peroxidation. Total lipid hydroperoxide content in oxidized membranes was determined iodometrically, using cumene hydroperoxide as the standard (9). Peroxidation of unsaturated lipids (except cholesterol) was also assessed by TBA assay in which malonaldehyde and other carbonyl by-products of peroxide decomposition react with TBA to produce colored adducts absorbing maximally at 532 nm. BHT was included to minimize autoxidation of unreacted lipids during the assay. Other details were as described earlier (6).

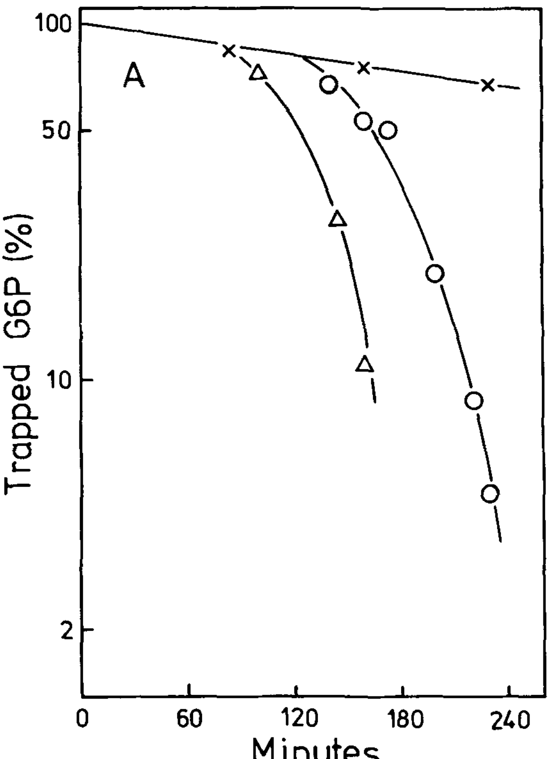
Chromatography of cholesterol oxidation products. Cholesterol products in photooxidized and xanthine/xanthine oxidase–treated membranes were analyzed by thin layer chromatography (TLC). The approach was adapted from previously published methods (10,11). Aliquots of 0.5 ml (0.45 mg total lipid) from reaction mixtures were mixed with 5 μl of 20 mM EDTA and extracted with 0.8 ml of chloroform/methanol (2:1, v/v) in polypropylene microfuge tubes. Retention of chelated iron in the aqueous phase minimized the possibility of hydroperoxide degradation during isolation. (Manipulations were done in subdued light when photosensitizers were present.) After centrifugation, 0.4 ml of the organic phase was transferred to a second microfuge tube. Reduction of the hydroperoxides to their relatively stable alcohols was accomplished by adding 25 μl of 40 mM $NaBH_4$ in methanol/10 mM NaOH. After 15 min incubation at room temperature, the solvent was evaporated by heating at 50 C under nitrogen. Up to three identical 0.4-ml samples (ca. 0.24 mg cholesterol) were combined during this step and stored at -20 C until analyzed. Borohydride reduction of 7-ketocholesterol yielded the epimeric 7α- and 7β-alcohols, which were used as standards. Each residue was dissolved in 10 μl of chloroform/methanol, applied to a Silica Gel-60 plate (EM Science, Cherry Hill, NJ) and chromatographed,

using heptane/ethyl acetate (1:1, v/v) as the solvent system. A single irrigation gave a satisfactory separation of the 5α-, 7α- and 7β-alcohols. Phospholipids remained at the origin in this system and therefore did not interfere with cholesterol product identification. After chromatography, plates were sprayed with 50% sulfuric acid and warmed briefly at 100 C to accelerate color development. In addition to cholesterol diols, partially separated hydroperoxide precursors (in nonreduced samples) could be detected; however, TMPD proved a more sensitive indicator for the latter. Immediately after color development, plates were photographed and in some instances scanned densitometrically, using a Hoefer instrument set in the reflectance mode.

RESULTS

Photosensitized vs oxidase-catalyzed lipid peroxidation and lysis. Resealed erythrocyte ghosts were studied as a model system for comparing the membrane-damaging effects of lipid peroxidation due to singlet oxygen and oxygen radical attack. Photoactivated UP was used as a source of $^1O_2$ (4), while xanthine oxidase acting on xanthine in the presence of iron was used as a source of O_2^-, H_2O_2 and other forms of partially reduced oxygen (6). Neither UP nor xanthine oxidase interact with the membrane, so that reactive species are initially generated in the medium rather than in the membrane per se. Time courses of lipid peroxidation (total lipid hydroperoxide [LOOH] formation) and lysis (accelerated release of G6P marker) were compared. Previous studies showed that increased G6P permeability under conditions of oxidative stress is correlated with lipid peroxidation damage (6,9,11). As shown in Figure 1A, continuous irradiation of UP-sensitized ghosts caused lysis after a lag period, the length of which depended on the porphyrin concentration. Dark or light controls containing or lacking porphyrin, respectively, released marker at the basal rate (5–10% per hr). LOOH content increased linearly throughout photooxidation, with no apparent lag (Fig. 1B). The threefold increase in rate upon going from 2 to 10 μM UP is approximately equal to the decrease in lag time to incipient lysis. At both concentrations of UP, lysis began when the LOOH level reached 11–13 nmol/mg protein (12–14 nmol/mg lipid).

The situation with xanthine/xanthine oxidase was more complex. Lysis commenced after a 2-hr lag, which could be shortened by adding H_2O_2 (0.1 mM) at the outset (Fig. 2A). Previous studies showed that lytic effects in this system are stimulated by iron and prevented by superoxide dismutase, catalase and chelators (e.g., EDTA, desferrioxamine), signifying O_2^- and H_2O_2 involvement via the iron-catalyzed Haber-Weiss cycle (6). On the other hand, photosensitized lysis by UP or PP showed no significant iron dependency and was not inhibited by catalase or superoxide dismutase (4,9). In contrast to photosensitized lipid peroxidation (Fig. 1B), xanthine/xanthine oxidase/iron-driven peroxidation showed a definite lag, which was shortened by supplementing with H_2O_2 (Fig. 2B). LOOH only began to accumulate after 30–60 min; a maximum level was



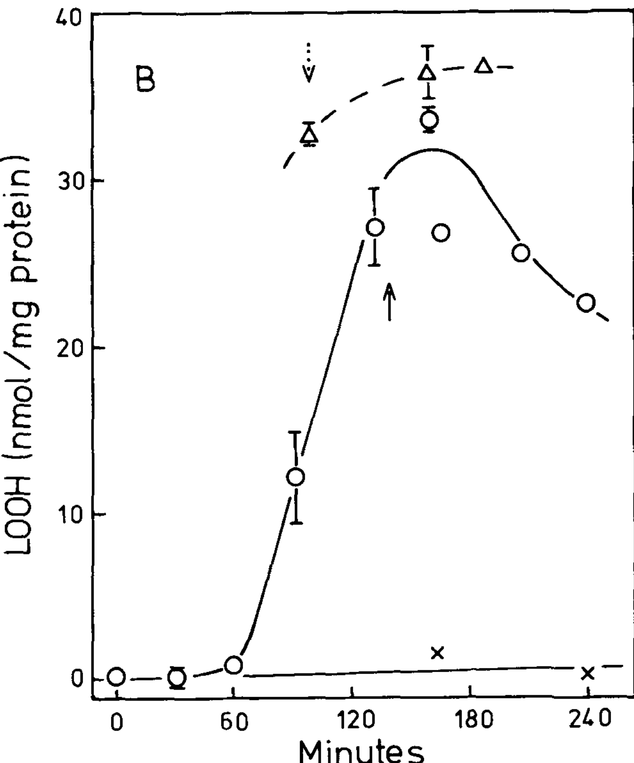


FIG. 2. Lipid peroxidation and lysis of resealed ghosts treated with xanthine, xanthine oxidase and iron. Membranes (1.0 mg protein/ml in PBS at 37 C) were incubated in the presence of xanthine oxidase (0.01 unit/ml), xanthine (1.0 mM), NaN_3 (0.1 mM) and $FeNH_4(SO_4)_2$ (0.1 mM) in the absence (○) and presence (Δ) of added H_2O_2 (0.1 mM). A control lacking oxidase and H_2O_2 was run alongside (×). (A) Membrane lysis (glucose-6-phosphate [G6P] release); (B) lipid hydroperoxide (LOOH) formation. Error bars show means ± deviations of values from duplicate determinations. Approximate time of incipient lysis is indicated by dotted arrow for H_2O_2 supplemented system and solid arrow for nonsupplemented system.

reached at about the time of lysis (150 min), followed by a decline. The LOOH content at incipient lysis (non-supplemented and H_2O_2-supplemented system) was 29–33 nmol/mg protein (32–36 nmol/mg lipid), i.e. 2–3 times higher than that observed in the photoreactions.

To gain additional insights into the mechanisms of photosensitized vs oxidase-catalyzed lipid peroxidation, we carried out these reactions in the presence of the phenolic antioxidants BHT and DTP. Both of these compounds are known to be excellent free radical traps. However, BHT may also react with or quench $^1O_2$ in competition with cholesterol and unsaturated fatty acyl groups (3). DTP on the other hand, is a relatively inefficient $^1O_2$ interceptor, making its use as a radical trap in the photodynamic system less problematic (3). As shown in Table 1, BHT at 10 μM (1–2 mol% of membrane phospholipid or cholesterol) strongly inhibited TBA-detectable lipid peroxidation in the xanthine/xanthine oxidase system and (not shown) prevented the formation of free radical oxidation products of cholesterol (see below). In contrast, 50 μM BHT failed to inhibit UP-sensitized peroxidation, but caused a small, albeit significant, decrease in the PP-sensitized reaction. DTP also inhibited the PP reaction (e.g., 35% at 2 hr), arguing in favor of radical (as opposed to $^1O_2$) trapping. Based on these results, a radical-mediated component (type I process) is apparent in the PP reaction, which may be favored by the ability of this relatively hydrophobic porphyrin to interact with the membrane (4). On the other hand, no free radical chemistry is apparent in the case of UP, which does not interact due to its high negative charge. Earlier studies (4) showed that azide inhibits UP- as well as PP-sensitized peroxidation, suggesting substantial $^1O_2$ intermediacy in each case.

Cholesterol oxidation products. Definitive evidence regarding oxidative mechanisms was sought by studying cholesterol oxidation. Cholesterol is converted to discrete products, the identification of which may allow $^1O_2$ and oxygen radical involvement in any given reaction to be distinguished (14). The obvious advantage is that this marker is an endogenous membrane lipid. As shown by the TLC results in Figure 3, xanthine/xanthine oxidase treatment of ghosts gave two cholesterol hydroperoxides whose borohydride reduction products comigrated with cholest-5-en-3β,7α-diol (7α-OH; R_f 0.15) and cholest-5-en-3β,7β-diol R_f 0.20), the epimeric pair associated with free radical oxidation (13,14). (Authentic 7β-OH; and 7β-OH were generated from 7-ketocholesterol and were distinguished from each other by the preponderance of the 7β-component.) No reaction occurred in the absence of xanthine oxidase or xanthine. The yield of 7α-OH and 7B-OH increased between 1 and 2 hr of incubation, slightly more of the latter being present at each time point. Densitometric comparison with known amounts of 7-OH indicated that <1% of the cholesterol was converted to these products. An additional component (R_f 0.37) has not yet been assigned. Catalase and superoxide dismutase prevented formation of these products when added individually to xanthine/xanthine oxidase reaction mixtures (Fig. 3). Thus, cholesterol oxidation is $O_2^{\cdot-}$- and H_2O_2-dependent, as previously reported for phospholipid peroxidation in this system (6).

The results of PP-sensitized photoxidation are also shown in Figure 3 for the sake of comparison. In this case a unique product of $^1O_2^-$- attack on cholesterol was

TABLE 1

Effects of Phenolic Antioxidants on Photodynamic and Xanthine Oxidase–Catalyzed Lipid Peroxidation[a]

System[a]	Additions	ΔA532 15 min	30 min	60 min	90 min	120 min
UP	None	0.016	0.045	0.104	0.140	—
	BHT, 50 μM	0.016	0.046	0.103	0.110	—
PP	None	0.037	0.073	0.143 ±0.005	0.192 ±0.010	—
	BHT, 50 μM	0.028	0.056	0.112	0.146	—
	DTP, 50 μM	0.022	0.045	0.091	0.125	—
X/XO/Fe	None	—	0.144 ±0.003	0.282 ±0.004	0.362 ±0.004	0.364 ±0.019
	BHT, 10 μM	—	0.009	0.021	0.032	0.043
	BHT, 50 μM	—	0.005	0.011	0.014	0.016

[a]Unsealed membranes were treated with xanthine (X)/xanthine oxidase (XO)/iron or irradiated in the presence of uroporphyrin (UP) or protoporphyrin (PP). Membrane protein concentration was 1 mg/ml, corresponding to ca. 0.7 mM phospholipid or 0.6 mM cholesterol in the bulk suspension. Other details are as indicated in Figures 1 and 2. Compensatory amounts of ethanol, the solvent used for BHT and DTP, were added to the reaction mixtures lacking these antioxidants. Lipid peroxidation was monitored in terms of TBA reactivity. Absorbance readings are corrected for zero-time values of controls not exposed to photooxidation or XO. (ΔA values for controls were 0.01 after 2 hr.) Experiments with PP (1 μM) were run at 10 C and with UP (10 μM) at 37 C. —, Not determined. Numbers with error limits are means ± deviation of values for duplicate determinations.

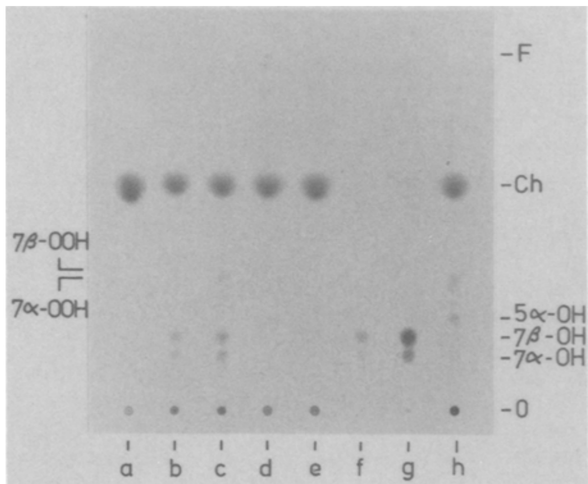


FIG. 3. Thin layer chromatography of cholesterol oxidation products from xanthine/xanthine oxidase/iron–treated ghosts. Extracted lipid hydroperoxides were reduced with $NaBH_4$. The standard reaction mixture (Std) was prepared as specified in Fig. 2. Lane a, Std − oxidase (120 min); b, Std (60 min); c, Std (120 min); d, Std + superoxide dismutase, 25 μg/ml (120 min); e, Std + catalase, 50 μg/ml (120 min); h, products of PP-sensitized photooxidation (cf Fig. 5). Sample loads (as starting cholesterol), 114 μg. Lanes f and g represent 7α-OH/7β-OH from 7-ketocholesterol, 0.8 μg and 8.0 μg respectively. Spots developed with 50% H_2SO_4 had the following colors: blue (5α-OH, 7α-OH, 7β-OH); magenta (Ch); gray (component between 5α-OH and Ch). Cholesterol 7α- and 7β-hydroperoxides in nonreduced samples were detected with N,N,N',N'-tetramethyl-p-phenylenediamine on a separate plate run alongside (positions shown with arrows). O, origin; F, solvent front.

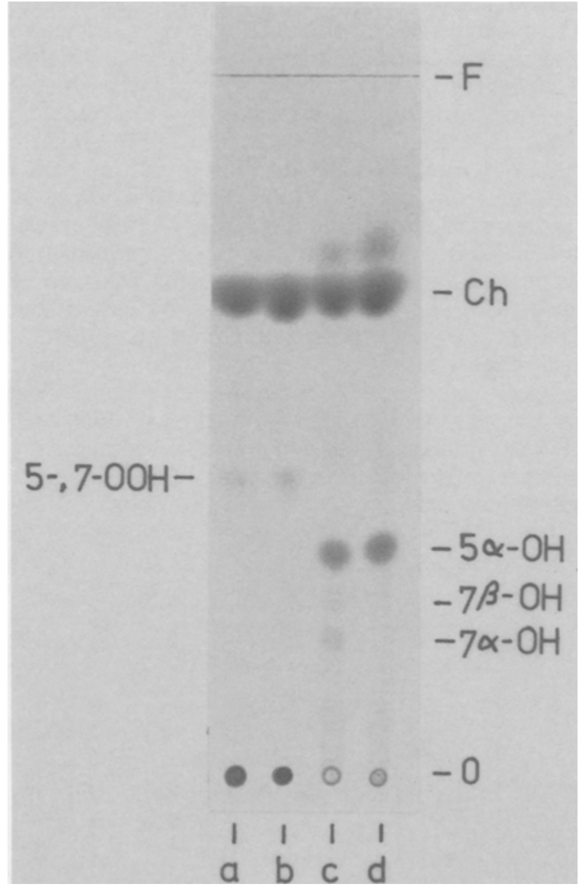


FIG. 4. Uroporphyrin- and protoporphyrin-sensitized photooxidation of membrane cholesterol. Thin layer chromatographic analysis was carried out after irradiating unsealed ghosts (1 mg protein/ml in phosphate-buffered saline at 37 C) for 1 hr in the presence of 5 μM PP (lane B) and 3 hr in the presence of 25 μM UP (lane C). Fluence rate, 20 mW/cm². Lane D shows a dark control (3 hr, no porphyrin) and lane A the 7-OH epimers from 7-ketocholesterol. Samples were reduced with borohydride before applying. The plate was scanned densitometrically immediately after developing. R_f values are as follows: 7α-OH, 0.13; 7β-OH, 0.19; 5α-OH, 0.23; Ch, 0.61. Ch peaks are deceptively small relative to diol peaks due to different color and lower absorptivity of Ch.

generated, namely, 3β-hydroxy-5α-cholest-6-ene-5-hydroperoxide (5α-OOH). A major component after borohydride reduction was more mobile (R_f 0.25) than the 7-OH epimers. It comigrated with material generated by rose bengal, a well-known $^1O_2$ sensitizer (data not shown), and on this basis was identified as 5α-OH. The absence of 5α-OH in xanthine oxidase–treated ghosts rules out any $^1O_2$ intermediacy in this reaction.

Additional evidence for the involvement of $^1O_2$ in photochemical peroxidation is provided in Figure 4, which shows densitometric scans of cholesterol products from PP- and UP-sensitized reactions. A significant 5α-OH peak was observed with each porphyrin, along with smaller amounts of the 7-OH epimers (7α-OH predominating). The latter might have arisen via the contributions of (i) type I (radical) photochemistry; (ii) general thermal decomposition of accumulating type II hydroperoxides; (iii) allylic decay of 5α-OOH to the more stable 7-OOH pair (15); or some combination of these pathways. The inability of BHT to protect against UP-sensitized phospholipid peroxidation (Table 1) argues against the first two possibilities in the case of this porphyrin. With PP, but not with UP, a component of similar color (gray) and mobility (R_f 0.35–0.37) to one seen with xanthine oxidase was observed (cf Fig. 3), and on this basis is ascribed to free radical reactions. (The component was shown not to be PP itself or oxidized PP.) This evidence, coupled with the partial inhibition of PP-driven lipid peroxidation by BHT or DTP (Table 1), supports our argument that PP photooxidizes partially via a type I mechanism.

To further clarify any type I contribution, we irradiated PP-sensitized ghosts at 10 C instead of 37 C in an effort to minimize thermally driven rearrangement of 5α-OOH (cf ref. 15). As shown in Figure 5 (lane c), 7α-OH and 7β-OH were still observed under these conditions after reduction of the hydroperoxides. Photooxidation in the presence of BHT strongly inhibited formation of the 7-OH epimers while not affecting 5α-cholest-6-ene-3β,5-diol (5α-OH) (lane d), as would be expected if the LOOH precursors are generated as described. The persistent spot of slightly greater mobility than cholesterol (lanes c and d) may represent dehydration product(s) of initially formed $^1O_2$ adduct (16).

Cholesterol photooxidation in the presence of ascorbate. As shown recently (4) and confirmed here (Table 2), peroxidation of polyunsaturated lipids in photosensitized ghosts is amplified markedly when irradiation is carried out in the presence of ascorbate (AH^-), a well-known electron donor. This is a new example of how AH^- (usually considered an antioxidant) can, under suitable conditions, act as a prooxidant. When

present at a low concentration (0.4 mM) at the start of a PP-sensitized reaction (Table 2), AH^- produced after 1 hr a nearly 10-fold increase in TBA reactivity over that observed without AH^-. Peroxidation was relatively insignificant in dark controls (minus or plus AH^-). EDTA abolished AH^--stimulated peroxidation, probably by removing membrane-bound iron that is required for redox cycling (4,9). BHT also inhibited the reaction, more so than it did basal photoperoxidation (Table 1); this is attributed mainly to quenching of alkoxyl radicals involved in propagation reactions (1,2). Stimulation was minimal at high concentrations of AH^- (e.g., 10 mM), presumably because antioxidant effects become more important.

To gain additional insights into the reaction mechanisms involved in these effects, we tracked cholesterol oxidation in the experiments described in Table 2. Samples were analyzed directly (Fig. 6A) or after borohydride reduction (Fig. 6B). As shown in Figure 6A, 0.4 mM AH^- caused striking changes, both qualitative and quantitative, in the cholesterol product profile of irradiated ghosts. Note that the major hydroperoxides appearing as a faint, poorly resolved doublet after 1 hr (lane d) are intensified with AH^- (lane f) and that the 7-OH epimers (undetectable in lane d) are now present in large amounts. Barely any 5α-OH is observed. In the presence of 10 mM AH^-, no hydroperoxides and much less of the 7-OH epimers are seen (lane h), which is consistent with the TBA results. Overall product yield was also less when BHT (lane i) or EDTA (lane j) was included along with 0.4 mM AH^-. In both cases peroxides were again apparent, and with EDTA no diols were seen. Borohydride reduction provided additional information (Fig. 6B). It is clear that $^1O_2$-driven peroxidation in the absence of AH^- shifted predominantly to free radical–mediated peroxidation in the presence of this reductant (compare lanes f and d). Moreover, inhibition of the AH^--stimulated reaction by BHT or EDTA was reflected in decreased amounts of 7α-OH/7β-OH and an increased amount of 5α-OH, the primary $^1O_2$-derived product (compare lanes f, i and j). Ascorbate may have diminished the yield of $^1O_2$ in this system, for example, by competing with ground state oxygen for PP triplet (17). Clearly, however, it could not have abolished $^1O_2$ formation, since considerable 5α-OH was detected when EDTA or BHT was present.

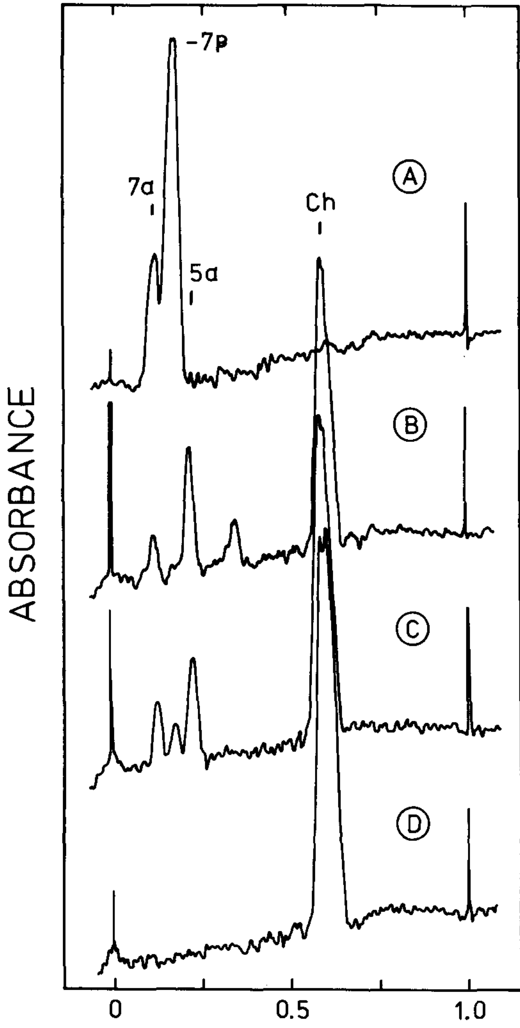


FIG. 5. Protoporphyrin (PP)-sensitized photooxidation of cholesterol: effect of butylated hydroxytoluene (BHT). Membranes were sensitized with 5 μM PP and irradiated for 2 hr at 10 C in the absence (lanes b and c) and presence (lanes a and d) of BHT (50 μM). The control mixture was adjusted to the same final concentration of ethanol (25 μM) that was introduced with BHT. Samples c and d were reduced with borohydride before chromatographing, while a and b were not reduced.

DISCUSSION

The cholesterol product analyses described in this work have provided important information about oxidative mechanisms in situ. Although cholesterol oxidation has been studied extensively in organic solvents and artificial membranes (14,18,19), very little has been done in natural membrane systems. The major obstacles encountered are limited material and low reaction rates. For example, $^1O_2$ reacts slowly with cholesterol ($k \sim 7 \times 10^4\ M^{-1}\ sec^{-1}$) compared with histidine ($k \sim 5 \times 10^7\ M^{-1}\ sec^{-1}$) (3,20). This makes product detection difficult and often requires

TABLE 2

Effects of Ascorbate on Protoporphyrin-Photosensitized Lipid Peroxidation[a]

	A_{532}		
Reaction system	10 min	30 min	60 min
Control (− AH^-)	—	—	0.055
Control (+ 0.4 AH^-)	—	0.050	0.058
Standard	0.048	0.068	0.114
+ 0.4 AH^-	0.075	0.293	0.615
+ 0.4 AH^-/EDTA	0.027	0.036	0.051
+ 0.4 AH^-/BHT	0.054	0.090	0.181
+ 10 AH^-	0.049	0.083	0.126

[a]The standard reaction mixture consisted of unsealed ghosts (1.0 mg protein/ml), $FeCl_3$ (25 μM) and PP (5 μM) is PBS at 37 C. Additional components in different experiments were as follows: AH (0.4 mM or 10 mM, as indicated); EDTA (0.1 mM); and BHT (0.03 mM). Suspensions were irradiated continuously (fluence rate 7.5 mW/cm^2), and samples were drawn for TBA assay at the indicated times. Nonirradiated controls (standard mixtures minus or plus AH^-) were run alongside. Zero-time A_{532} was 0.049 (0.025 for EDTA-containing system). —, Not determined.

the use of radiolabeled sterol (21). By analyzing large amounts of easily obtained material, we were able to determine cholesterol products directly, without having to resort to radiolabeling. Although the results are mainly qualitative, they demonstrate for the first time, for example, that a reductant like AH^- can switch $^1O_2$-dependent peroxidation to a free radical–dominated process, which greatly enhances the damaging effects of $^1O_2$ alone. In addition, the free radical nature of xanthine oxidase–catalyzed lipid peroxidation in cell membranes (6) has been verified by showing that 7α-OH and 7β-OH are formed. Recent evidence (22) that $^1O_2$ is not among the damaging oxidants produced by this enzyme was corroborated, ruling out earlier suggestions to the contrary.

By examining cholesterol products and the effects of phenolic antioxidants, we conclude that highly polar UP, which does not bind to the membrane, photosensitizes lipid peroxidation and lysis by a type II mechanism (reactions 1 and 2, where ^3UP is triplet excited state porphyrin and LH is unsaturated lipid).

$$^3UP + O_2 \rightarrow UP + {}^1O_2 \quad [1]$$

$$LH + {}^1O_2 \rightarrow LOOH \quad [2]$$

Although PP-sensitized photooxidation also involves $^1O_2$, a small part appears to be type I (radical) in nature. The reaction pathway in this case could be as follows:

$$^3PP + LH \rightarrow PP^{-\cdot} + H^+ + L^{\cdot} \quad [3]$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad [4]$$

$$LOO^{\cdot} + LH \rightarrow LOOH + L^{\cdot} \quad [5]$$

Reaction 3 depicts initiation of peroxidation via electron transfer, and reactions 4 and 5 show propagation. Autoxidation of $PP^{-\cdot}$ might also contribute to initiation by generating partially reduced oxygen species (17). Whether other relatively hydrophobic sensitizers will behave similarly in this membrane system remains to be established.

The results shown in Table 2 and Figure 2 suggest that in vivo phototoxic effects of lipid peroxidation might be influenced by AH^- or other cellular reductants. The following mechanism can be envisaged.

$$AH^- + Fe(III) \rightarrow A^{-\cdot} + H^+ + Fe(II) \quad [6]$$

$$LOOH + Fe(II) \rightarrow LO^{\cdot} + OH^- + Fe(III) \quad [7]$$

Cholesterol or phospholipid hydroperoxides generated by $^1O_2$ attack (reaction 2) undergo iron-catalyzed reduction to alkoxyl radicals (reactions 6 and 7), which then trigger rounds of free radical peroxidation (1,2,4). Thus, AH^- amplifies lipid peroxidation via 1-electron reduction of nascent photoperoxides. Molecular oxygen probably competes with LOOH for Fe(II) in reaction 7, in which case H_2O_2-dependent (Fenton) formation of strongly oxidizing $OH^{\cdot}$ might occur. We have found, however, that catalase not only fails to inhibit AH^--stimulated peroxidation (UP-sensitized), but consistently stimulates it (unpublished observation), indicating that the effect is not H_2O_2-dependent. Since $LO^{\cdot}$ is necessarily generated in the lipid matrix, it would not be surprising to find that this radical is a better initiator of peroxidation than hydroxyl.

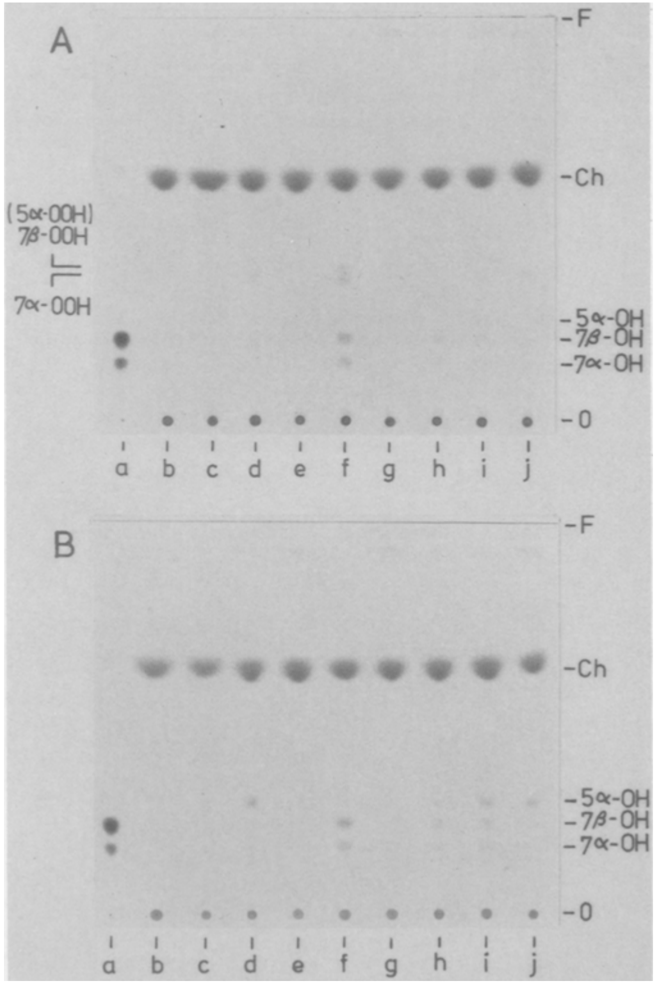


FIG. 6. Effects of ascorbate on protoporphyrin-sensitized photooxidation of cholesterol. Lipid extracts from the experiments described in Table 2 were analyzed by thin layer chromatography before (A) and after (B) borohydride reduction. Samples (0.16 mg in starting cholesterol) were analyzed after 10 min and 60 min of irradiation: standard reaction mixture (Std), 10 min (c) and 60 min (d); Std + 0.4 mM AH^-, 10 min (e) and 60 min (f); Std + 10 mM AH^-, 10 min (g) and 60 min (h); Std + 0.4 mM AH^-/0.03 mM BHT, 60 min (i); Std + 0.4 mM AH^-/0.1 mM EDTA, 60 min (j). A 60-min dark control is shown in lane b and authentic 7α-OH, 7β-OH from 7-ketocholesterol in lane a.

It is interesting that resealed ghosts treated with xanthine/xanthine oxidase/iron began to lyse (release G6P more rapidly) when their LOOH content was more than twice that of UP-photooxidized ghosts (Figs. 1 and 2). Therefore, the structural damage necessary to produce a marker-releasing pore appeared to be greater with the xanthine oxidase system. There is a qualitative difference in some of the hydroperoxides produced in systems of this type (Fig. 3; refs. 14,24,25), and it is possible that at any given LOOH level, enzymatically generated peroxides perturbed the bilayer less than photogenerated peroxides. Another possibility is suggested by our findings (unpublished) that xanthine oxidase treatment causes membrane aminolipids to be cross-linked, whereas photooxidation does not. Crosslinking could impede marker efflux, making it necessary for a greater fraction of lipid to be oxidized before net efflux occurs.

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METHODS

Loss of NADPH During Assays of HMG-CoA Reductase: Implications and Approaches to Minimize Errors

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In assays of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, preincubation of isolated washed microsomes with NADPH led to a time- and protein concentration–dependent loss of enzyme activity. This occurred despite the presence of an NADPH regenerating system. Addition of fresh NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase restored activity. Of the individual components, only NADP was effective. Errors due to loss of NADPH are most pronounced in assays using high microsomal protein, low NADPH levels and preincubation with NADPH and when glutathione rather than dithiothreitol is present. To minimize the effects of NADPH depletion, it is recommended that (i) NADP and NADPH not be present during the preincubation period; (ii) incubation periods be relatively short; (iii) microsomal protein concentrations be less than 1 mg; and (iv) NADPH concentrations be 1 to 2 mM.
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With the recognition that purified preparations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase represented a proteolytic fragment containing the active site rather than the intact enzyme (1), a shift back to studying the reductase within the microsomal membrane has occurred (2,3). Assay conditions, however, were developed to maximize the activity of the proteolytically modified form of the enzyme. For example, it has become standard practice to preincubate for 20 min with NADPH to reactivate this cold-sensitive enzyme (4). The native form of HMG-CoA reductase is not cold-liable (5). It also has been common to use 2–5 mM NADPH and 10 mM dithiothreitol in HMG-CoA reductase assays. However, it recently has been demonstrated that HMG-CoA reductase displays allosteric kinetics with respect to NADPH concentrations (3) and that the enzyme exists in both a disulfide-linked dimer and a free sulfhydryl monomer (2). Addition of 10 mM dithiothreitol can convert dimer to monomer (2). In view of these observations, reductase assays using lower NADPH concentrations and thiol conditions more closely approximating those found in vivo are desirable.

In an effort to identify a specific degradative system for HMG-CoA reductase, liver microsomes from rats fed colestipol were incubated with those from fasted rats. Although a marked decrease in mevalonate formation occurred, this did not result from inactivation of the reductase. Rather, as the data presented in this report show, a loss of NADPH occurred. The consequences of NADPH depletion and approaches to minimize its effects are addressed.

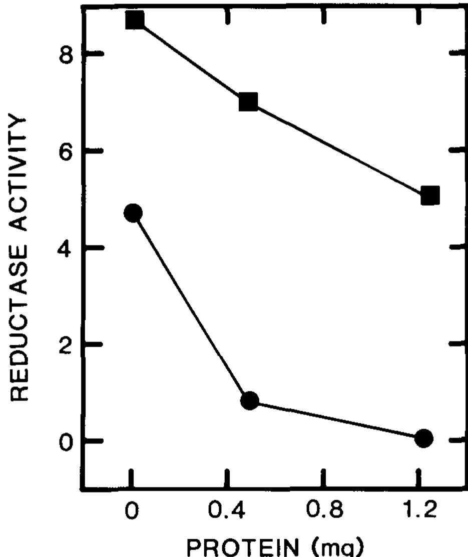

FIG. 1. Effect of adding hepatic microsomal protein from a fasted rat to HMG-CoA reductase reactions containing microsomes from a colestipol-fed rat. Reaction mixtures were either preincubated (●) or not (■) for 20 min at 37 C. All reactions contained 100 mM potassium phosphate buffer, pH 7.1; 200 mM potassium chloride; 5 mM reduced glutathione; 4 mM glucose 6-phosphate; 500 μM NADP$^+$; 0.5 units of glucose 6-phosphate dehydrogenase 70 μM RS[^{14}C]HMG-CoA (3,700 cpm/nmol) and 0.24 mg of liver microsomal protein from a colestipol-fed rat. Reaction mixture volumes were 300 μl. Reactions were started by the addition of HMG-CoA (●) or by adding microsomes that had been warmed to 37 C (■). Incubation was carried out at 37 C for 10 min. Reactions were stopped by the addition of 30 μl of 2.4 N HCl. Reductase activity is given as nmol of mevalonate formed for duplicate determinations.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 125 to 150 g were purchased from Harland Industries (Madison, WI). Animals were maintained on 2% cholesterol or 2% colestipol and 0.04% mevinolin or were fasted 48 hr prior to being killed at the diurnal high point of the daily cycle (6). Lysosome-free microsomes were prepared in 0.25 M sucrose as previously described (6). HMG-CoA reductase activity was assayed by the radiochemical method using thin layer chromatography (TLC) for isolation of the lactonized product (7). Colestipol and mevinolin were gifts from Upjohn (Kalamazoo, MI) and Merck (Rahway, NJ), respectively.

RESULTS AND DISCUSSION

As shown in Figure 1, addition of increasing amounts of fasted microsomes decreased HMG-CoA reductase

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Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; TLCK, Nα-p-tosyl L-lysine chloromethyl ketone; TLC, thin layer chromatography; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetracetic acid; G6P, glucose 6-phosphate; G6PD, glucose 6-phosphate dehydrogenase.

activity in microsomes from colestipol-fed animals. This was most pronounced in the series where microsomes were preincubated. It is also apparent that preincubation of microsomes from colestipol-fed animals without adding microsomes from fasted rats significantly reduced reductase activity. Addition of various proteinase inhibitors, such as leupeptin, ethylene glycol bis (β-aminoethyl ether) (EGTA) and Nα-p-tosyl L-lysine chloromethyl ketone (TLCK) failed to prevent the decrease in reductase activity, suggesting that a proteinase might not be responsible for the loss of activity.

The possibility that the observed loss of reductase activity might not be unique to fasted microsomes was examined in the experiment depicted in Figure 2. Adding increasing amounts of liver microsomes from colestipol-fed rats also resulted in a decline in reductase activity that was dependent on preincubation time and amount of microsomal protein added.

Table 1 illustrates that the loss in reductase activity that occurs during preincubation results from a loss in the NADPH regenerating system. Adding fresh NADP, glucose 6-phosphate (G6P) and glucose 6-phosphate dehydrogenase (G6PD) (complete system) at the end of the preincubation period restored reductase activity. However, this activity is somewhat less than that seen in reactions started by adding both NADPH and HMG-CoA. Preincubation of microsomes for 20 min without NADPH does not inactivate, but rather stimulates reductase activity. Increasing the amount of NADPH in the preincubation mixture twofold does not offset the problem. As shown in Table 2, the complete NADPH regenerating system was more effective that any single component in restoring reductase activity. Of the individual components, only NADP was effective. The data indicate a nearly complete loss of NADP and a partial loss of G6P occurred during preincubation. These losses could not be prevented by addition of EDTA, liver cytosol or microsomal phosphatase inhibitors (8) fluoride or bovine serum albumin. Presumably, loss of NADPH would not occur in vivo. It is of interest that the loss of NADPH is not nearly as serious in reactions in which 10 mM dithiothreitol was used rather than 5 mM glutathione (Fig. 3).

Depletion of NADPH during preincubation and likely during the incubation period would be expected to have profound effects on studies of HMG-CoA reductase activity. As shown in Figure 4, preincubation with NADPH can result in serious errors in determination of the allosteric kinetics of HMG-CoA reductase. It is possible that the previously reported (3,7) allosteric

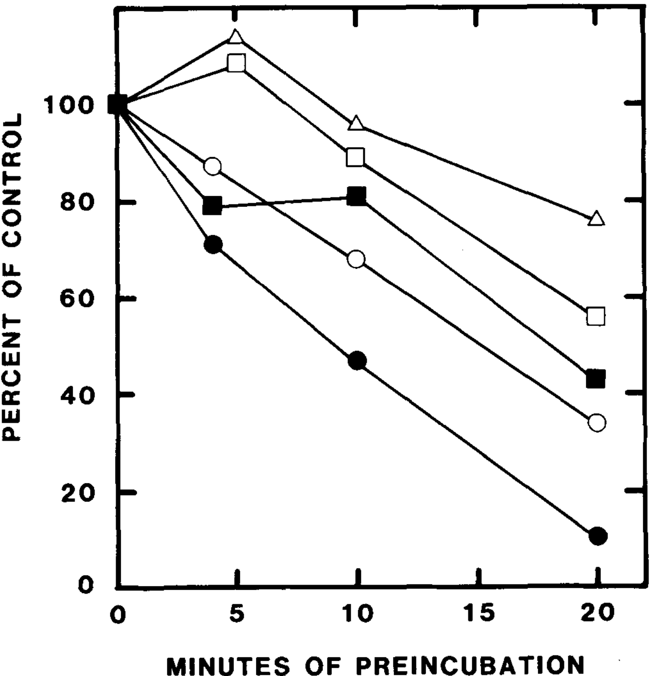

FIG. 2. Effect of microsomal protein concentration on loss of HMG-CoA reductase activity during preincubation with NADPH. Increasing amounts (0.15 mg [Δ], 0.30 [□], 0.50 [■], 0.70 [○] and 0.90 [●]) of microsomal protein from a colestipol-fed rat were preincubated for the indicated times in reaction mixtures as described in the legend to Fig. 1. Reactions were started by the addition of RS[^{14}C]HMG-CoA and incubated for 10 min at 37 C. Reductase activity is expressed as a percentage of that at zero min of preincubation for each series. Reductase activities in terms of nmol of mevalonate formed for the non-preincubated controls are 2.44 (Δ), 4.15 (□), 5.08 (■), 5.71 (○) and 6.12 (●).

TABLE 1

Decrease in HMG-CoA Reductase Activity During Preincubation, Apparently Resulting from Loss of the NADPH Regenerating System

Additions at 0 min	Additions at 20 min	Reductase activity (nmol/min/mg)
Microsomes, NADPH[a]	HMG-CoA	0.01
Microsomes, NADPH	NADPH, HMG-CoA	0.63
	Microsomes, NADPH, HMG-CoA	0.66
Microsomes	2× NADPH, HMG-CoA	1.52
Microsomes	NADPH, HMG-CoA	0.94
Microsomes, 2× NADPH	HMG-CoA	0.01

All reactions were preincubated for 20 min and incubated for 5 min at 37 C. Reaction mixtures contained 1 mg of microsomal protein from a colestipol-fed rat and components listed in the legend to Fig. 1. Values are the averages of duplicate determinations.

[a]NADPH refers to addition of NADP, glucose 6-phosphate and glucose 6-phosphate dehydrogenase.

TABLE 2

Effect of Individual Components of the NADPH Regenerating System on Restoration of HMG-CoA Reductase Activity Lost During Preincubation

Additions	Reductase activity (nmol/min/mg)
None	0.02
Complete	0.95
NADP	0.55
G6P	0.04
G6PD	0.07
NADP + G6P	0.73
NADP + G6PD	0.53

G6P, glucose 6-phosphate; G6PD, glucose 6-phosphate dehydrogenase. All reactions were preincubated for 18 min at 37 C and contained 0.9 mg of microsomal protein from a colestipol-fed rat and all the components listed in the legend to Fig. 1, including the NADPH regenerating system. Additions at 18 min were NADP, 0.5 mM; G6P, 4 mM and G6PD, 0.5 units (complete) or the indicated components. Two min later the reactions were started by addition of RS[^{14}C] HMG-CoA and incubated for 5 min. Values are the averages of duplicate determinations.

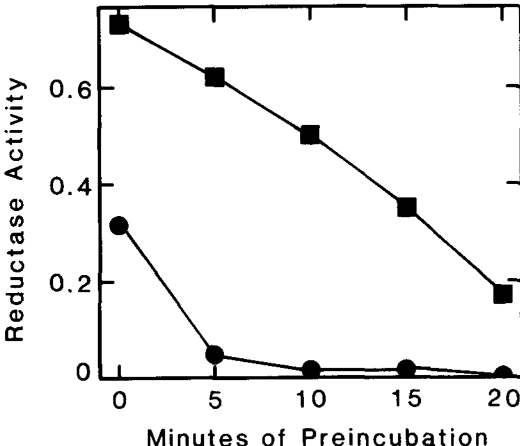

FIG. 3. Effect of thiol on loss of NADPH during preincubation. Microsomes (2.3 mg from a colestipol-fed rat) were preincubated for the indicated times with 5 mM glutathione (●) or 10 mM dithiothreitol (■) with the complete NADPH system described in the legend to Fig. 1 (500 μM NADPH). Reactions were started by the addition of [^{14}C]HMG-CoA. Reactions were stopped after 5 min. Reductase activity is given as nmol/min/mg.

NADPH-dependent kinetics (3,7) are either entirely due to NADPH depletion or are markedly affected by this problem. It is not possible to distinguish between these possibilities because of the need to use low NADPH concentrations and the lack of linearity with time at such levels.

One approach to the problem of NADPH depletion is simply to increase its concentration in reaction mixtures. However, substrate inhibition at high concentrations of NADPH has been reported for HMG-CoA reductase (9). We confirmed this. Generally, inhibition was observed at NADPH concentrations above 4 mM. In microsomes from rats fed mevinolin and colestipol, inhibition was apparent at 1 mM. The NADPH concentration at which inhibition occurred was decreased by incubation with higher concentrations of thiols, i.e., 10 mM dithiothreitol.

Recognition of the NADPH depletion problem allows formulation of more appropriate conditions for assaying native microsomal HMG-CoA reductase, as shown in Figure 5. Central to these conditions are deletion of a preincubation step, low microsomal protein concentrations, short incubation times and adequate NADPH concentration. Under these conditions, good linearity with time can be demonstrated even with 5 mM glutathione as the thiol present.

Over the years, numerous potential pitfalls in assaying HMG-CoA reductase activity have been identified. These include the nonspecificity of the anion exchange assay (10); presence of interfering activities, such as HMG-CoA lyase (11,12) and mevalonate kinase (13); proteolysis (1); and inhibition by R HMG-CoA (14). To this list, we now add microsomal depletion of NADPH.

It would appear that the NADPH-depleting activity is a constitutive microsomal activity. When high concentrations of microsomal protein are used to assay HMG-CoA reductase activity in conditions where the level of enzyme is low, an underestimation of activity can readily result. This could lead to erroneous conclusions concerning changes in enzyme levels caused by dietary and hormonal agents or in correlations with changes in rates of cholesterol synthesis.

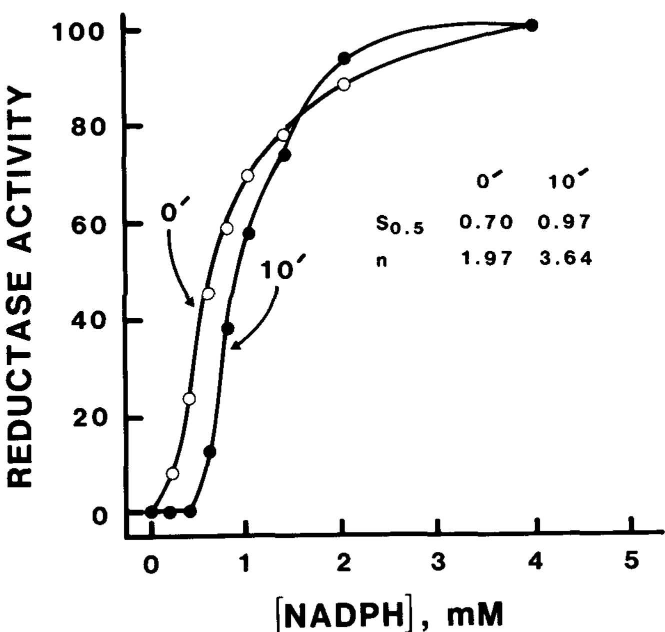

FIG. 4. Effect of preincubation on the allosteric kinetics of HMG-CoA reductase. Microsomes (0.22 mg) from a colestipol-fed rat were either preincubated for 10 min (●) or not (○) with the complete NADPH system. The reactions were started by the addition of [^{14}C]HMG-CoA. Reductase activity is given as a percentage of the maximal activity, 1.43 (●) and 0.92 (○) nmol/min/mg, respectively. The Hill coefficients (n) and $S_{0.5}$ values obtained from Hill plots are given. Reaction mixture components are as described in the legend to Fig. 1 except that the glucose 6-phosphate concentration was 20 mM.

More accurate determinations of microsomal HMG-

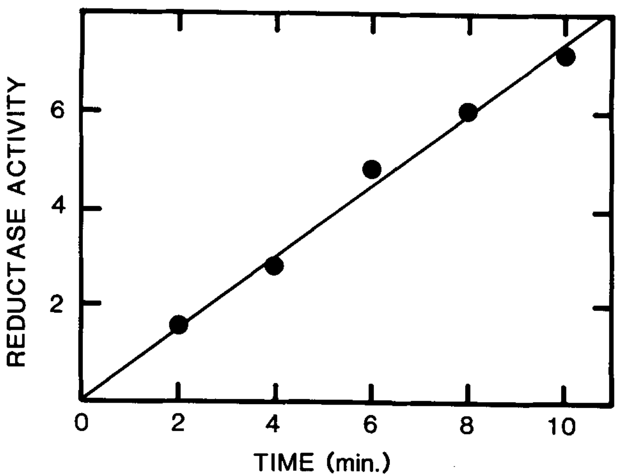

FIG. 5. Linearity of microsomal HMG-CoA reductase activity with incubation time. Reaction mixture components were as listed in the legend to Fig. 1 except that the concentration of NADP$^+$ was 1.5 mM, and 0.20 mg of microsomal protein from a rat fed colestipol was present in each reaction mixture. The microsomes were warmed at 37 C with glutathione, potassium phosphate buffer and potassium chloride for 10 min. The reactions were started by adding a mixture containing the NADP$^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, RS[^{14}C]HMG-CoA and 2 mM potassium phosphate buffer, which was warmed at 37 C for 5 min prior to addition. Reductase activity is given as nmol of mevalonate formed.

CoA reductase activity should aid in determining whether changes in rates of synthesis and degradation of HMG-CoA reductase mRNA and protein (15–18) or in the extent of posttranslation modifications such as phosphorylation (19) or sulfhydryl status (20) can totally account for a given change in enzyme activity.

ACKNOWLEDGMENTS

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Analysis of Lipids Containing Hydroxy Fatty Acids

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Lipids containing hydroxy fatty acids or hydroxyacyl moieties are acetylated with [1-^{14}C]acetic anhydride or [^{3}H]acetic anhydride, and the content of hydroxy fatty acids or hydroxyacyl moieties is estimated from the specific radioactivity of the acetylated products with respect to that of radioactive standards, such as radioacetylated ricinoleic acid or triricinoleoylglycerol. Mixtures of triacylglycerols containing one, two and three hydroxyl groups per molecule are derivatized in a similar manner, and the resulting acetates are fractionated by thin layer chromatography according to the number of acetate groups per molecule. The relative proportion of each type of triacylglycerols in the mixture is estimated from the distribution of radioactivity in the various fractions. Applications of these techniques are demonstrated by the analysis of several seed lipids.
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Lipids containing hydroxy fatty acids occur widely in animals, plants and microorganisms (1,2). Several analytical techniques have been developed for the qualitative and quantitative analysis of hydroxy fatty acids and lipids containing hydroxyacyl moieties (3,4). Among these techniques, gas liquid chromatography (GLC) has been used most extensively. However, quantitative GLC of methyl esters of hydroxy fatty acids poses several problems, such as long retention times of these substances and relative instability at high temperatures (3). The quantitative analysis of lipids containing hydroxy fatty acids becomes especially difficult when highly reactive acids, such as dimorphecolic or kamlolenic acids, are present. Some of these problems can be partially solved by derivatization of the hydroxyl groups (3).

In the present communication, we describe procedures for the analysis of lipids containing hydroxy fatty acids or hydroxyacyl moieties. These procedures are based on isotopic derivative techniques, partly in conjunction with thin layer chromatography (TLC).

MATERIALS AND METHODS

Castor *(Ricinus communis)* beans were obtained from the Department of Botany, Aligarh Muslim University, Aligarh, India. *Wrightia tinctoria* seeds were supplied by the National Botanical Research Institute, Lucknow, India. *Grevillea decora* seeds were a gift from R. Kleiman, U.S. Department of Agriculture–Agricultural Research Service, Peoria, Illinois. Seeds of *Mallotus philippinensis, Onguekoa gore, Dimorphotheca aurantiaca, Artemisia absinthium* and *Strophanthus kombe* were provided by L. J. Morris, now at Unilever Research, Colworth House, Sharnbrook, Bedfordshire, England.

All reagents, solvents and adsorbents were products of E. Merck A.G. (Darmstadt, Federal Republic of Germany). Solvents were distilled before use. [1-^{14}C]Acetic anhydride (2 mCi/mmol) and [^{3}H]acetic anhydride (50 mCi/mmol) were purchased from NEN Chemicals GmbH (Dreieich, Federal Republic of Germany). TLC was carried out on glass plates (20 × 20 cm) coated with a 0.3-mm layer of Silica Gel H.

Thin layer chromatograms were assayed for radioactivity by means of a Berthold scanner LB 2760 (BF-Vertriebsgesellschaft, Wildbad, Federal Republic of Germany). The radioactivity of silica gel scrapings and lipid samples was determined using a Packard Tri-Carb C 2405 liquid scintillation spectrometer (Packard Instruments Co., Downers Grove, Illinois) using ^{14}C- or ^{3}H-labeled hexadecane as internal standard.

Lipids were extracted under nitrogen from finely ground seeds either with hexane at room temperature or with chloroform/methanol (2:1, v/v) as described elsewhere (5). Castor bean lipids were fractionated by TLC using hexane/diethyl ether/acetic acid (50:50:1, v/v/v) to isolate diricinoleoylacylglycerols and triricinoleoylglycerol. Saponification of lipids, removal of nonsaponifiable matter and liberation of fatty acids were carried out according to established procedures (6). Ricinoleic acid was isolated from the total fatty acids derived from castor bean lipids by TLC using hexane/diethyl ether/acetic acid (50:50:1, v/v/v) as developing solvent.

Radioactive acetic anhydride, diluted to desired specific radioactivity and dissolved in twice its volume of anhydrous pyridine, was used for acetylation. Lipid samples (50–100 mg) were reacted under nitrogen in screw-capped tubes with 1 ml of the acetylating mixture at room temperature overnight or at 50–60 C for 2 hr. After cooling to room temperature, the reaction mixture was diluted with 5 ml water, and the acetylated lipids were extracted three times with 3 ml each of hexane. The combined hexane extracts were washed three times each with 3 ml of 10% sulfuric acid, 3 ml of 10% aqueous sodium bicarbonate and finally with water. The solvent was evaporated, and residual traces of water were removed azeotropically with benzene/chloroform/methanol (1:1:1, v/v/v). The samples were weighed and dissolved in a known volume of hexane, and the radioactivity in aliquots was measured by liquid scintillation counting in Econofluor (NEN Chemicals).

An aliquot (10–20 mg) of the acetylated castor bean lipids was applied as a streak on a chromatoplate, which was then developed with hexane/diethyl ether/acetic acid (60:40:1, v/v/v). The lipid fractions were stained with iodine vapor and identified by cochromatography with unlabeled standards. The radioactively labeled fractions were located by scanning and scraped off. To the silica gel scraping, 100 µl water and 10 ml Aquasol-2 (NEN Chemicals) were added, and radioactivity was measured by liquid scintillation counting. All data reported are means of triplicate determinations.

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RESULTS AND DISCUSSION

The methods reported here involve acetylation of lipids with radioactive reagents, such as [1-^{14}C]acetic anhydride or [^{3}H]acetic anhydride, followed by determination of the specific radioactivity of the acetylated products. The content of hydroxy fatty acids and/or hydroxyacyl moieties in the lipids is estimated by comparison of the specific radioactivity of the acetylated products with those of the corresponding isotopically labeled acetates of pure hydroxy fatty acids or their esters. Furthermore, radioactively labeled acetates derived from mixtures of lipids containing free hydroxyl groups are resolved by TLC into fractions differing by the number of acetate (corresponding to hydroxyl) groups per molecule. The distribution of radioactivity in the various fractions is determined to estimate the composition of such lipid mixtures.

Initially some standard samples containing known proportions of hydroxy fatty acids or hydroxyacyl moieties were acetylated with ^{14}C- or ^{3}H-labeled acetic anhydride. A blank of pure oleic acid was also treated with radioactive acetic anhydride to check whether traces of radioactivity remained in the samples after removal of excess reagent. The percentage of hydroxy fatty acid or hydroxyacyl moieties was estimated from the specific radioactivities of the corresponding acetates derived from ricinoleic acid and triricinoleoylglycerol, respectively. The data show a close agreement between the known values and those found experimentally (Table 1).

The hydroxy fatty acid content of a few selected seed lipids was determined by acetylation of the total lipids with [1-^{14}C]acetic anhydride/pyridine reagent of different specific activities. The content of hydroxy fatty acids plus hydroxyacyl moieties was calculated from the specific radioactivities of the acetylated products with reference to the specific radioactivity of the acetate derived from triricinoleoylglycerol as standard. It was found that essentially similar results are obtained with acetic anhydride reagents of various specific radioactivities. However, the sensitivity of the method increases with increasing specific radioactivity of the reagent (Table 2).

The percentage of hydroxy fatty acids plus hydroxyacyl moieties determined by radioacetylation of the total lipids of several seeds and the values reported in the literature are compared in Table 2. A good agreement is observed between these values in several cases.

In the lipids of *M. philippinensis* the value observed is much lower than that reported, most likely because large proportions of the hydroxyl groups of kamlolenic (18-hydroxyoctadeca-9,11,13-trienoic) acid present in these lipids are esterified as estolides (8). The lipids of *D. aurantiaca* contain a conjugated dienol, i.e., 9-hydroxy-octadeca-10,12-dienoic acid. Because of the reactive nature of the conjugated dienol structure, the percentage of this hydroxy fatty acid plus hydroxyacyl moieties in the total lipids has been determined by countercurrent distribution of the methyl esters of the total fatty acids (9). The percentage of hydroxy fatty acid obtained by

TABLE 1

Quantitative Estimation of Hydroxy Fatty Acids or Hydroxyacyl Moieties in Standard Mixtures Prepared from Ricinoleic and Oleic Acids and in Triacylglycerols

Reagent/lipids	Specific activity (dpm/mg)	Hydroxy fatty acids/ hydroxyacyl moieties (%)
[1-^{14}C]Acetic anhydride	1,493	
Ricinoleic acid (20%) + oleic acid (80%)	187	20.8
Ricinoleic acid (10%) + oleic acid (90%)	91	10.1
Ricinoleic acid (standard)	899	100
Oleic acid (blank)	0	0
[^{3}H]Acetic anhydride	33,293	
Diricinoleoylacylglycerols	4,350	66.4
Triricinoleoylglycerol (standard)	6,549	100

TABLE 2

Estimation of Hydroxy Fatty Acids (HFA) in Seed Lipids by Acetylation with [1-^{14}C]Acetic Anhydride of Different Specific Activities

Reagent/lipids	Sp act of reagent/ acetylated lipids (dpm/mg)	HFA (%)	Sp act of reagent/ acetylated lipids (dpm/mg)	HFA (%)	Sp act of reagent/ acetylated lipids (dpm/mg)	HFA (%)	HFA (%) reported in literature (ref)
[1-^{14}C]Acetic anhydride	547,000		49,326		2,858		
Ricinus communis	72,082	85.6	6,364	85.6	365	85.6	89 (7)
Mallotus philippinensis	19,367	23.0	1.899	25.5	105	24.8	72 (8)
Dimorphotheca aurantiaca	54,863	65.1	4,953	66.6	276	65.2	65 (9)
Onguekoa gore	41,651	49.4	3,813	51.3	226	53.3	36 (10)
Artemisia absinthium	38,769	45.7	3,505	47.1	201	47.5	7 (11)
Strophanthus Kombe	18,487	22.0	1,753	23.6	99	23.5	14 (12)

TABLE 3

Estimation of Hydroxy Fatty Acids in Mixtures of Fatty Acids Obtained by Saponification of Seed Lipids

		Hydroxy fatty acids (%)	
Reagent/lipids	Specific activity (dpm/mg)	Observed	Reported in literature (ref)
[1-^{14}C]Acetic anhydride	1,493		
Ricinus communis	1,458	85.4	89 (7)
Wrightia tinctoria	1,173	68.7	70 (13)
Grevillea decora	207	12.1	~10 (14)
Ricinoleic acid (standard)	1,708	100	

TABLE 4

Analysis of Triacylglycerols of Castor Bean Lipids Containing One, Two and Three Hydroxyl Groups per Molecule

	dpm		Composition (%)	
Lipids	Total	Corrected[a]	Observed	Reported in literature (ref. 7)
Triricinoleoylglycerol	265.8	88.6	69.3	68.2
Diricinoleoylacylglycerols	60.7	30.35	23.7	23.1
Monoricinoleoyldiacylglycerols	2.38	2.38	1.8	2.9
Dihydroxystearoylricinoleoylglycerols	19.1	6.39	5.0	4.9

[a]Calculations were made considering the number of hydroxyl groups per molecule.

radioacetylation (Table 2) is in close agreement with the literature value (9). In the seed lipids of *A. absinthium*, the value of hydroxy fatty acids plus hydroxyacyl moieties found is much higher than reported in the literature (11). This is obviously due to the presence of 23% epoxyacyl moieties (11), which react with acetic anhydride as well.

It is evident that the isotopic derivative technique described here is not a reliable method if part of the hydroxy fatty acids occur as estolides or if epoxides are present. Moreover, the presence of other hydroxy compounds, such as sterols, monoacylglycerols and diacylglycerols, which also react with acetic anhydride, can affect the results. Most of these problems were overcome by saponifying the seed lipids to cleave the estolide linkages and removing the unsaponifiable matter. The resulting mixed fatty acids were subjected to radioacetylation, and the hydroxy fatty acid content was determined from the specific radioactivity of the resulting acetates with reference to the specific radioactivity of the acetate derived from ricinoleic acid as standard. The results obtained with the lipids from three seed species show close agreement with values reported in the literature (Table 3).

The procedures described here also permit the analysis of mixtures of triacylglycerols containing different numbers of hydroxyl groups per molecule. The results of an analysis of the lipids of castor *(R. communis)* bean are presented to demonstrate the potential of the isotopic derivative technique in conjunction with TLC. Lipids of castor bean were reacted with [1-^{14}C]acetic anhydride/pyridine reagent, and the resulting acetylated lipids were fractionated by TLC. The relative proportions of the various lipid classes in the total mixture were determined from the ratio of radioactivities in these fractions, corrected in terms of the number of hydroxyl groups per molecule. The results shown in Table 4 agree well with those reported in the literature (7).

Isotopic derivative techniques have so far received limited attention for the analysis of lipid mixtures (15-20). High sensitivity and simplicity of these methods make them ideally suited for the analysis of lipids containing hydroxyl groups.

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Specialty Lipids and Their Biofunctionality

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Medium Chain Triglycerides and Structured Lipids

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Lipids are an essential component of our body composition and necessary in our daily food intake. Conventional fats and oils are composed of glycerides of long chain fatty acids and are designated as long chain triglycerides (LCT). Body fat as well as the fats and oils in our daily intake fall into this category. In enteral and parenteral hyperalimentation, we can identify such LCT fats and oils. Soy, corn, safflower and sunflowerseed oils are typical of the LCT oils.

In the search for alternative noncarbohydrate fuels, medium chain triglycerides (MCT) are unique and have established themselves in the areas of malabsorption syndrome cases and infant care and as a high energy, rapidly available fuel. Structure lipids with a MCT backbone and linoleic acid built into the triglyceride molecule have been developed to optimize the triglyceride structure that is best for patients, particularly the critically ill. Structured lipids with built-in essential fatty acid components or other polyunsaturated fatty acids promise greater flexibility in patient care and nitrogen support.
Lipids 22, 417-420 (1987).

Recent years have brought a renewed interest in lipids and their role in the metabolic and dietetic applications in health care of hospitalized patients as well as the public at large (1-33; Shah, N.M., and Iber, F.L., private communication).

This symposium focuses on a specific, unique segment of the lipid picture: the medium chain triglycerides (MCT) and structure lipids prepared from them.

A review and summary is presented here to lay the groundwork for the papers that follow. Each presentation supplements the broad picture that is evolving from the animal and human research that is progressing at various universities and laboratories.

REVIEW AND DISCUSSION

Fats and oils of animal, vegetable and marine origin have a fatty acid spectrum that ranges from C_2-C_{24}, with variations in not only physical and chemical characteristics but also in isomers and positional structure in the triglyceride molecule. Most fats and oils are composed of long chain fatty acids and are termed long chain triglycerides (LCT). Dairy fat, meat fat and vegetable oils fall into this category (Table 1).

The lauric fats, however, are composed primarily of fatty acids of C_{14} chain length and shorter. Coconut and palm kernel oils are typical of this class of lauric fats. They represent the main source of the C_8-C_{10} acids required for synthesis of MCT (Table 2).

MCT are different from all of the fats and oils we conventionally use. Where conventional fats and oils are absorbed via the lymphatic system and are carnitine-dependent for chylomicron formation and transport, MCT are absorbed via the portal system, are not carnitine-dependent and do not require chylomicron formation. The metabolic pathways of the MCT and LCT distinguish the unique aspects of the MCT (Tables 3 and 4; Fig. 1).

Aside from the transport difference, MCT demonstrate certain additional characteristics of considerable advantage. Whereas lipids generally are slowly absorbed and metabolized and energy is expended to oxidize and utilize them as fuel or building blocks, MCT are absorbed and

TABLE 1

Typical Long Chain Triglyceride Oils

	Type of oil				
Fatty acid	Corn	Peanut	Safflower	Soybean	Sunflowerseed
Lauric					0.5
Myristic		0.1	0.1	0.1	0.2
Palmitic	12.2	11.6	6.5	11.0	6.8
Palmitoleic	0.1	0.2		0.1	0.1
Margaric		0.1			
Stearic	2.2	3.1	2.4	4.0	4.7
Oleic	27.5	46.5	13.1	23.4	18.6
Linoleic	57.0	31.4	77.7	53.2	68.2
Linolenic	0.9			7.8	0.5
Arachidic	0.1	1.5	0.2	0.3	0.4
Gadoleic		1.4			
Eicosadienoic		0.1			
Behenic		3.0		0.1	
Lignoceric		1.0			

TABLE 2

Typical Lauric Fats and Oils

	Type of oil				
Fatty acid	Babassu	Coconut	Cohune	Palm kernel	Tacum
Caproic	0.4	0.5	0.3	0.3	0.2
Caprylic	5.3	8.0	8.7	3.9	2.9
Capric	5.9	6.4	7.2	4.0	2.3
Undecanoic			0.1		
Lauric	44.2	48.5	47.3	49.6	51.8
Myristic	15.8	17.6	16.2	16.0	22.0
Palmitic	8.6	8.4	7.7	8.0	6.8
Stearic	2.9	2.5	3.2	2.4	2.3
Oleic	15.1	6.5	8.3	13.7	9.3
Linoleic	1.7	1.5	1.0	2.0	2.4
Arachidic	0.1	0.1		0.1	

TABLE 3

Medium Chain Triglyceride Oil Specifications

Free fatty acids (as oleic)	0.05% max
Saponification value	345-355
Iodine value (Wijs)	1.0 max
Acetyl value	5.0 max
Setting point	−5 C
Color (Lovibond)	10 yellow/1.0 red
Unsaponifiables	0.5 max
Fatty acid composition	
C_6 1-2%	
C_8 65-75%	
C_{10} 25-35%	
C_{12} 2% max	

metabolized as rapidly as glucose while having better than twice the caloric density of protein and carbohydrate. They are easily oxidized and utilized as fuel and energy, with little tendency to deposit as body fat. For a quick, high energy source, MCT are outstanding.

These unique features of MCT have been recognized and utilized over the years. Oral supplementations and enteral feeding formulas of MCT products are available for use in a variety of areas, including care of infants, epileptic children and cystic fibrosis patients and for intestinal resection (35-37). For such established areas of MCT use, the practice has been to physically mix 15-20% of a highly polyunsaturated vegetable oil (to insure essential fatty acid requirements) with the MCT oil (38).

In the area of parenteral nutrition, we have been limited for many years to lipid emulsions based on soybean oil and now safflower oil (Table 5). Both are LCT types of lipid and pose a number of problems.

The current practice of using lipid emulsion in a total parenteral nutrition (TPN) regimen has been based on the need for a noncarbohydrate source for fuel and energy and the need to satisfy essential fatty acid requirements and/or deficiencies. LCT supply caloric needs and alleviate essential fatty acid deficiency. They have, however, shown a tendency to deposit as fat (a large proportion of the infused lipid) rather than to satisfy immediate fuel requirements. LCT lipid emulsions also are too slow in clearing from the blood and oxidize too slowly to supply fuel and energy.

At present, there is controversy over the optimum feeding regimen for the critically ill patient. An all-carbohydrate TPN system may promote visceral protein attraction and obligatory hepatic lipogenesis. There is no consensus, however, that lipid emulsions composed of LCT are optimal. There is concern that these emulsions are less than ideal because of a relative carnitine deficiency that occurs in sepsis, which blocks their entry into the mitochondria for β-oxidation. Numerous additional studies have shown reduced clearance of these emulsions in the critically ill patient and increased potential for elongation, desaturation and deposition in the liver and other organs.

In contrast, MCT have been shown to have a carnitine-independent entry into the mitochondria, to have a more rapid β-oxidation and to be less likely to undergo elongation and deposition (39-42). Emulsions composed

TABLE 4

Rationale for Use of Medium Chain Triglycerides (MCT)

Physicochemical characteristics	Physiologic considerations	Potential therapeutic applications
MCT present more interfacial surface for enzyme action/unit time	Intraluminal enzymatic hydrolysis of AMCT is more rapid and complete than LCT	Decreased intraluminal concentrations of pancreatic lipase (pancreatic insufficiency, cystic fibrosis) Decreased small-bowel absorptive surface (intestinal resection)
Greater water solubility of MCT hydrolysis products	Bile salts are not required for dispersion in water	Decreased intraluminal concentrations of bile salts (intrahepatic and extrahepatic biliary-tract obstruction, chronic parenchymal liver disease)
Smaller molecular size of MCT vs LCT	Small amounts of MCT may enter intestinal cell without prior hydrolysis	Pancreatic insufficiency
Shorter chain length of fatty acids derived from MCT	More efficient penetration of diseased mucosal surface	Nontropical sprue, tropical sprue
Small molecular size and lower pK of fatty acids derived from MCT	Intramucosal metabolism of MCFA different from LCFA: Decreased affinity for esterifying enzymes Decreased affinity for activating enzymes Minimal reesterification of MCFA to MCT No chylomicron formation	Abeta-lipoproteinemia Hypobeta-lipoproteinemia
Greater water solubility of MCFA	Different routes of transport of MCT vs LCT: Portal transport of MCT (as MCFA) Lymphatic transport of LCT (as chylomicrons)	Lymphatic obstruction (lymphomas) Intestinal lymphangiectasia

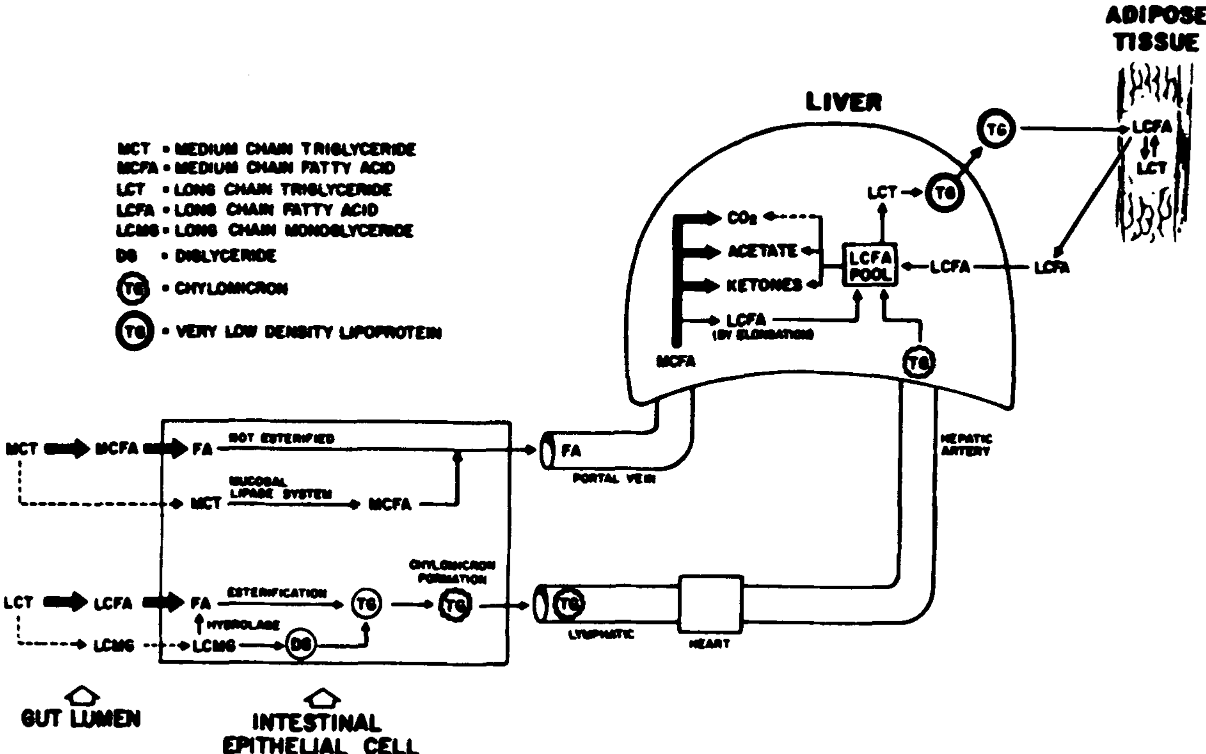

FIG. 1. Transport of medium and long chain triglycerides.

TABLE 5

Fatty Acid Composition of Oils for Parenteral Emulsions

Fatty acid	Type of oil		
	Soybean	Safflower	MCT
6:0	—	—	<2
8:0	—	—	70
10:0	—	—	30
12:0	—	—	<2
14:0	0.1	0.1	—
16:0	10.5	6.7	—
18:0	3.2	2.7	—
18:1	22.3	12.9	—
18:2	54.5	77.5	—
18:3	8.5	tr	—
20:0	0.2	0.5	—
20:1	0.9	0.5	—

$[C_8, C_8, C_8]$ + $[C_{18=}, C_{18=}, C_{18=}]$ → $[C_8, C_8, C_8]$ $[C_{18=}, C_{18=}, C_{18=}]$

Mixture
(nonrandom distribution)

$[C_8, C_8, C_8]$ $[C_8, C_8, C_{18=}]$ $[C_8, C_{18=}, C_{18=}]$ $[C_{18=}, C_{18=}, C_{18=}]$

Rearranged mixture
(random distribution)

FIG. 2. Physical mix vs rearrangement.

principally of MCT may offer a unique and readily available fuel for the injured and stressed patient (43). Also, since MCT readily undergo β-oxidation, ketonemia is usually much more pronounced than with LCT emulsions. Skeletal muscle can readily burn ketone bodies for fuel and may spare the oxidation of branched chain amino acids and reduce skeletal protein catabolism (44–52).

In an effort to develop the optimum lipid structure for parenteral use, we considered structured lipids using MCT and LCT having linoleic and/or linolenic fatty acids. The resulting rearranged triglycerides have both medium chain fatty acids and a polyunsaturated fatty acid on the same glycerine molecule. Based upon the molar ratios of the MCT and the LCT with a high unsaturated fatty acid, one can obtain the structured lipid of the desired combinations (Fig. 2). We have considered further the structured lipids with an MCT backbone by adding the essential fatty acid (linoleic acid) into the triglyceride molecule at various levels (45,46).

If LCT emulsions are too slow in clearing and suffer from other drawbacks, and MCT emulsions may be too rapid in clearing and suffer from the absence of essential fatty acids, then the structured lipid with sufficient linoleic acid to satisfy essential fatty acid needs will also serve to slow down the clearance of the MCT backbone to a more acceptable level (Table 6). A structured lipid

TABLE 6

Approximate Composition of Glycerides

	Captex 810 series structured lipids (%)			
	A	B	C	D
I MCT (3 short)	62	39	15	3
II MCT (2 short, 1 long)	32	43	40	20
III MCT (1 short, 2 long)	6	16	27	44
IV LCT (3 long)	<1	2	3	34

having about 25% linoleic acid (Captex 810B) appears to be suitable for such parenteral lipid emulsion use. Preclinical and clinical testing remains to supply the needed data to validate this concept. The animal studies to date are encouraging and show certain additional benefits for the use of such structured lipids in hyperalimentation. Further investigation is warranted.

The rationale for and concept of preparing structured lipids are gaining support of research laboratories. It appears feasible that triglycerides where at least one fatty acid is a polyunsaturated fatty acid and at least one is a medium chain fatty acid can be obtained in glyceride oils. The actual format and combination of the fatty acids on the glycerine molecule will have to be investigated. Whether we shall require specific structures of high purity or can take advantage of molecular rearrangement to yield a mixture of structures will be determined by the functionality of such compositions. It appears highly probable, however, that lipid compositions of medium and long chain glycerides will advance our application of such lipids, both for the treatment of patients and the general welfare of the public.

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Medium Chain Triglycerides and Structured Lipids as Unique Nonglucose Energy Sources in Hyperalimentation

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This brief review will discuss recent work concerning new intravenous lipid emulsions for future use in clinical patients. Intravenous lipid emulsions currently available in the United States are derived from soybean or safflower oils and serve as sources of nonglucose, nitrogen-sparing calories and the essential fatty acid linoleic acid. Because of concerns that much of the infused long chain triglyceride is not oxidized readily and that there may be some immune system impairment, newer emulsions utilizing medium chain triglycerides have been developed.
Lipids 22, 421–423 (1987).

Long chain triglycerides. Intravenous feeding of hospitalized patients has evolved rapidly. Before the availability of lipid emulsions suitable for intravenous use, glucose was the only nonprotein source of calories. Meeting the full caloric needs of the patient with glucose often led to hepatic lipogenesis and increased respiratory work to expire the excessive carbon dioxide produced during lipogenesis (1). Because intravenous fat emulsions made from soy or safflower oils contain linoleic acid, an essential fatty acid, their use was implemented. These long chain triglycerides (LCT) serve as a nonglucose fuel that provides energy so that the body can use amino acids as protein and not as a caloric source (2). Since fat burns at a lower respiratory quotient than glucose—that is, it produces less carbon dioxide for the same amount of oxygen uptake—this is a benefit for patients with pulmonary compromise having problems expiring all the CO_2 they are producing. In addition, long chain fatty acids will inhibit lipogenesis from carbohydrate, thereby decreasing fatty livers. In the diabetic patient, lipid calories decrease insulin requirements if substituted for glucose calories.

Essential fatty acids. The requirement for linoleic acid is met by supplying 4% of calories as the essential fatty acid. Since soybean oil is slightly more than one-half linoleic acid, supplying 10% of total calories as a soybean oil lipid emulsion easily meets essential fatty acid requirements. The issue of α-linolenic acid being essential has recently been raised by anecdotal reports as well as work by Neuringer et al. (3). Based on this work, it seems that long chain ω3 fatty acids, found in high concentrations in the brain and retina, are essential fatty acids. The primate work emphasizes their essentiality for infants but not necessarily for adults. Unlike safflower oil, soybean oil contains 7 or 8% α-linolenic acid, and since the current commercial lipid emulsions are derived from soybean oil in whole or in part, supplying these emulsions would probably meet requirements for both linoleic and α-linolenic fatty acids.

Immune system effects. Fischer et al. gave mice intraperitoneal Intralipid® and then followed that with the administration of intraperitoneal Streptococcus (4). In the lipid-treated group, there were significantly increased mortality and bacteremia and decreased neutrophil chemotaxis. Shaw and Wolfe were developing an *Escherichia coli* sepsis dog model and reported 100% mortality in the animals when 10% lipid was infused for 1 or 2 hr prior to intravenous injection of bacteria (5). The dose in these studies was ca. 115 mg per kg body weight per hr, a clinically relevant dose. Fraser and colleagues showed decreased chemotaxis of monocytes in patients as well as in normal subjects after the intravenous administration of 100 g of fat over 8 hr (6). The reticuloendothelial system (RES) is that collection of cells in the body concerned with phagocytosis of particulate matter in the bloodstream. It consists primarily of macrophages in the liver, spleen and bone marrow. A fourth study highlighting the clinical significance of a well-functioning RES was done by Rimola et al. (7). They studied 41 cirrhotic patients with routine clinical and laboratory parameters as well as liver-spleen scans and technetium-99 sulfur colloid clearance from the blood. They found that the only parameters that predicted mortality or the development of bacteremia were the tests of RES function such as the liver-spleen scans and technetium-99 sulfur colloid clearance rates. All other clinical and laboratory parameters were insufficiently sensitive to detect clinically vital outcomes such as the incidences of bacteremia and mortality.

A problem with LCT emulsions is slow clearance of the infused triglyceride from the bloodstream. Also, clearance is not synonymous with oxidation of the fatty acids, a primary purpose for using the emulsion. For these reasons, as well as the fact that medium chain fatty acids are rapidly cleared from the blood and are rapidly oxidized independent of carnitine transport and poorly stored in adipose tissue, medium chain triglyceride (MCT) emulsions were looked to for clinical intravenous use (8–10).

Medium chain fatty acid preparations. MCT oil consists essentially of octanoic and decanoic acids (8 and 10 carbons long, respectively), whereas soybean and safflower oil consist almost exclusively of fatty acids of 16 and 18 carbon chain length (Table 1). The fatty acids exist as triglycerides. In soybean and safflower oils, the fatty acids on the triglyceride are long chain; in MCT, they are

TABLE 1

Fatty Acid Composition of Selected Oils (As Percent of Total Fatty Acids)

Fatty acid	Soybean	Safflower	MCT
6:0			<2
8:0			70
10:0			30
12:0			<2
14:0	0.1	0.1	
16:0	10.5	6.7	
18:0	3.2	2.7	
18:1ω9	22.3	12.9	
18:2ω6	54.5	77.5	
18:3ω3	8.5		

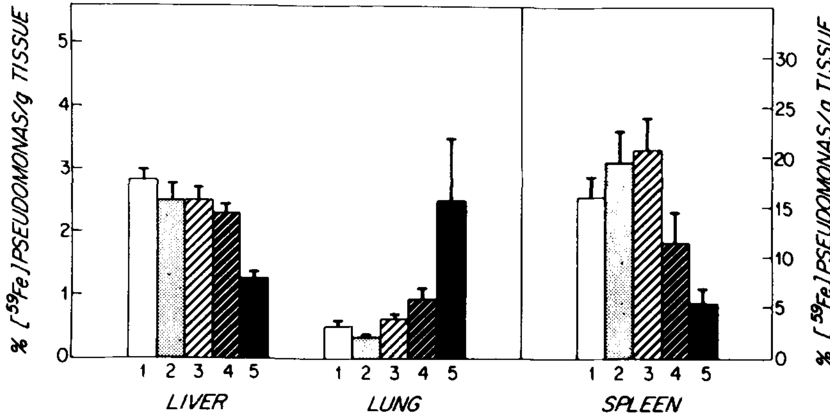

FIG. 1. Organ uptake of radiolabeled Pseudomonas following long chain triglyceride (LCT). Group 1, 100% glucose; group 2, 25% LCT/75% glucose; group 3, 50% LCT/50% glucose; group 4, 75% lipid/25% glucose; group 5, 100% LCT (ref. 11).

medium chain. A third group of triglycerides that has been investigated is that of structured lipids. These lipid molecules are triglycerides but are made from reesterified mixtures of MCT and LCT. The mixtures are hydrolyzed and allowed to reesterify randomly, thereby forming a triglyceride molecule of both medium and long chain fatty acids. As such, they are chemically distinct from physical mixtures of MCT and LCT.

Animal experiments. Our laboratory has recently published three experiments done on laboratory animals where these lipid emulsions were investigated. In the first experiment, rats were fed a total parenteral nutrition (TPN) regimen for three days (11). The rats had been given a 30% scald burn while anesthesized. At the end of three days, the animals were given a radiolabeled intravenous bacteremia; the clearance of this bacteremia into organs was measured. Rats were divided into groups that differed by the amount of fat (as LCT only) and glucose calories they received. It was shown that as the proportion of carbohydrate calories fell and that of lipid calories increased (the groups were equicaloric) there was a shift in the organ uptake of bacteria. Liver uptake decreased slowly with increasing lipid doses, whereas lung uptake of the radiolabeled bacteria increased markedly (Fig. 1). Next, different types of triglycerides at the same caloric proportions were given. The three lipids that were contrasted were LCT, MCT and structured lipid. LCT emulsions gave statistically increased lung uptake of the radiolabeled bacteria over that of the MCT and structured lipid groups (Fig. 2). Total caloric intake and proportion from fat calories were similar for these groups. This study emphasizes the fact that LCT blocks RES function whereas medium chain fatty acid-based lipid emulsions do not.

A second study was done by Hamawy et al. (12). Here, rats were given bilateral septic femoral fractures with the implantation of gauze containing *E. coli.* The animals were placed on TPN for four days and then studied. At the end of four days of TPN with septic fractures, blood was withdrawn and cultured to assess any bacteremia from the fractures. The group that received amino acids and dextrose but no lipid showed 10^3 bacteria/ml, as did

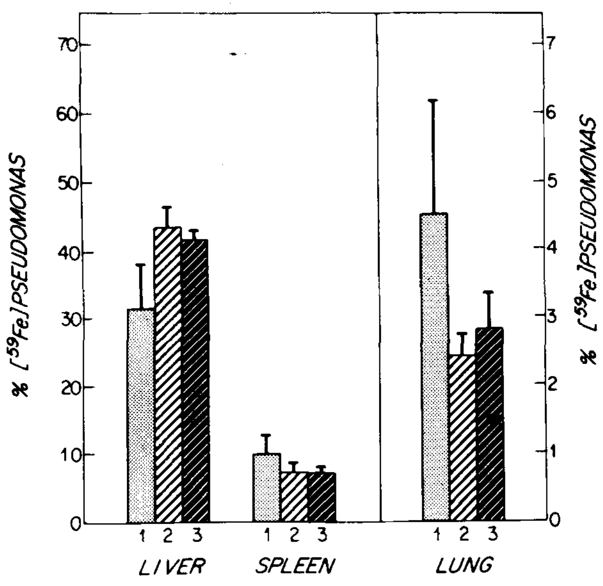

FIG. 2. Organ uptake of radiolabeled Pseudomonas following long chain (LCT), or medium chain triglyceride (MCT) or structured lipid. Group 1, LCT; group 2, MCT; group 3, structured triglyceride (ref. 11).

the group receiving one-third of its calories as LCT. The third group received a physical mixture of 75% MCT and 25% LCT as one-third of calories and showed no bacteremia (Fig. 3). Second, like in the experiment by Sobrado et al. described above, radiolabeled *E. coli* were injected into the blood. A statistically significant decrease in liver uptake of the *E. coli* bacteremia was seen in the LCT group compared with the 75% MCT and 25% LCT physical mixture group (Fig. 4). Inversely, in the lung, the MCT/LCT physical mixture group had a statistically significant lower uptake of bacteria compared with either the long chain or amino acid/dextrose TPN groups (Fig. 5). This study again highlights the significant difference in RES interference from intravenous lipid emulsions, with beneficial effects seen in the MCT/LCT physical mixture group.

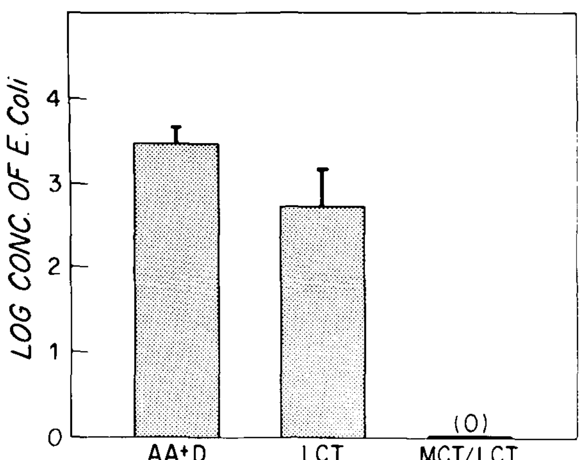

FIG. 3. Baseline bacteremia following septic fracture. AA + D, amino acids and dextrose; LCT, long chain triglyceride; MCT, medium chain triglyceride (ref. 12).

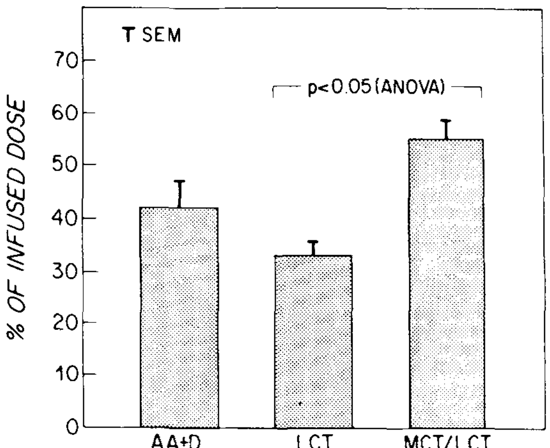

FIG. 4. Uptake of radiolabeled *E. coli* by the liver. AA + D, amino acids and dextrose; LCT, long chain triglyceride; MCT, medium chain triglyceride (ref. 12).

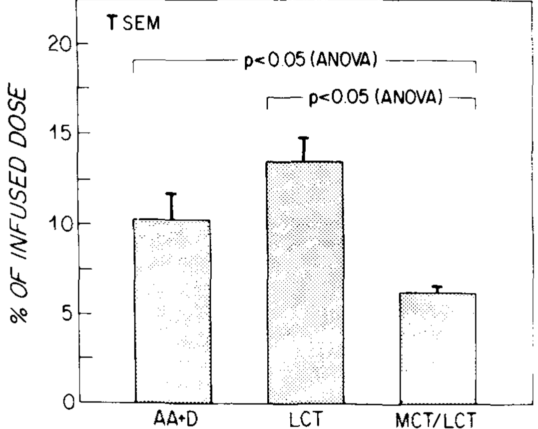

FIG. 5. Uptake of radiolabeled *E. coli* by the lung. AA + D, amino acids and dextrose; LCT, long chain triglyceride; MCT, medium chain triglyceride (ref. 12).

In the third study, by Mok et al. (13), rats also were given three days of TPN following a 25% scald burn done under anesthesia. The animals were divided into five groups according to TPN regimen. Group 1 was fed 200 calories per kg body weight per day as amino acids and dextrose and no lipid. Group 2 was fed ca. 300 calories/day, again consisting solely of amino acids and dextrose. Groups 3, 4 and 5 were fed ca. 300 calories/day with one-third of nonprotein calories as either LCT, MCT or structured lipid, respectively. The hypocaloric 200-kcal group and the MCT group lost weight during the experiment, whereas the other groups gained weight. This highlights the somewhat increased thermogenesis of MCT compared to other lipid fuels. Nitrogen balance did not differ significantly among the groups, but tended to show a higher balance in the structured lipid group. Albumin levels were measured and shown to be remarkably higher in the structured lipid group. This experiment shows the potential nitrogen-sparing benefits of structured lipid emulsions compared to other types of intravenous lipid.

Clinical data. We have begun to investigate intravenous MCT as a 75% MCT/25% LCT physical mixture in hospitalized patients. Preliminary analysis of the data shows that the fuels are safe and, as measured by serum triglyceride and free fatty acid analysis, are hydrolyzed and cleared rapidly. In addition, thermogenesis is noted in patients, but this was not accompanied by increased body temperature (14,15). European studies have shown similar results; they have had a 50/50 physical mixture of MCT and LCT available commercially for two years (16).

Studies to date support the notion that supplying all lipid calories in a TPN regimen as LCT is not the best nutritional care for patients. Giving a large proportion of these lipid calories as medium chain fatty acids, either as physical mixtures of MCT or as structured lipids, provides more readily oxidizable fuels with less interference of the reticuloendothelial component of the immune system.

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Absorption of Safflower Oil and Structured Lipid Preparations in Patients with Cystic Fibrosis[1]

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Patients with cystic fibrosis (CF) and pancreatic insufficiency usually have decreased linoleic and increased oleic, palmitoleic and eicosatrienoic (20:3ω9) acids compared to normal values of blood and tissue lipids. These changes are consistent with early essential fatty acid deficiency and are observed despite the regular use of exogenous pancreatic enzyme supplementation. As part of a study to determine the relative role of malabsorption as the etiology for the altered fatty acid status, the change in total plasma fatty acids and in area percent of plasma linoleic acid was determined in CF patients and control subjects following the ingestion of various lipid supplements, including two safflower oil preparations and two structured lipid preparations. Fasting subjects consumed 36 g of lipid in a milkshake containing 15 g of protein and 45 g of carbohydrate. Plasma samples obtained 0, 2, 4, 6 and 8 hr after the meal showed that the CF patients absorbed all preparations when administered with their regular dose of pancreatic enzyme supplement. Comparison of the patterns of increase for total plasma fatty acids and area percent of plasma linoleic acid following the administration of the different lipid supplements in CF patients and control subjects suggests that (a) malabsorption alone is not the cause of the abnormal fatty acid composition in the lipids of CF patients and that increased caloric intake along with consumption of adequate amounts of linoleic acid should improve the linoleic acid status of CF patients; (b) there may be selectively increased metabolism of certain fatty acids from the ingested lipids in the relatively malnourished CF patient compared to control subjects; and (c) conditions favoring the persistence of nonpancreatic lipases seem also to favor absorption and utilization of the structured lipid preparation containing medium chain length fatty acids and linoleic acid in CF patients compared to control subjects.
Lipids 22, 424–428 (1987).

It has been recognized by many investigators that patients with cystic fibrosis (CF) and pancreatic insufficiency have changes in the fatty acid composition of their blood and tissue lipids consistent with early essential fatty acid deficiency, despite regular pancreatic enzyme supplementation (1–7). More recently, CF patients without clinical symptoms of exocrine pancreatic insufficiency have been reported to have altered fatty acid compositions (8–11). These patients usually have decreased linoleic (18:2ω6) and increased oleic, palmitoleic and eicosatrienoic (20:3ω9) acids relative to normal values.

The decrease in plasma and tissue linoleic acid levels generally has been attributed to the malabsorption associated with exocrine pancreatic insufficiency observed in 85–90% of CF patients. Altered hepatic fatty acid metabolism, including a decrease in desaturase activity, also has been suggested (1,7,12,13). In addition, decreased linoleic acid levels could be due, in part, to a restriction of dietary intake or an increased metabolic requirement for linoleic acid in CF patients with inadequate caloric intake. Decreased caloric intake in association with increased caloric needs in CF patients (14–17) could lead to metabolism of linoleic acid to meet immediate energy needs and consequently decrease the amount available as an essential fatty acid for elongation to arachidonic acid, prostaglandin biosynthesis, incorporation into membrane phospholipids and other functions (3,18–20).

Previous investigations of long-term supplementation of linoleic acid derived from a variety of sources have had inconsistent results (3,7,21–23). Studies in which major emphasis was placed on compliance and awareness of total caloric intake have demonstrated a possible benefit (24–26). As part of a study to understand and estimate the relative role of malabsorption as the cause of the fatty acid alterations in CF patients and to evaluate the efficacy of linoleic acid supplementation, the increase in plasma total fatty acids and percent linoleic acid content was determined after ingestion of test meals containing various lipid supplements. The test was somewhat analogous to a glucose tolerance test. The results of linoleic acid absorption from these test meals have been reported previously (13). This report presents new data regarding the absorption of total fatty acids from the preparations and compares this data with previously reported linoleic acid absorption patterns observed in CF patients and control subjects for each of the lipid preparations studied.

MATERIALS AND METHODS

Subjects. Nine CF patients with exocrine pancreatic insufficiency (four female, five male, aged 14–38 years) and seven control subjects (one female, six male, aged 19–37 years) were studied. The CF patients had a medical history and clinical evaluation indicative of CF diagnosis and a positive sweat test. Exocrine pancreatic insufficiency had been determined previously for each of the CF patients. CF patients were taking multivitamin supplements, exogenous pancreatic enzymes and oral antibiotics (dicloxacillin, tetracycline or trimethoprim-sulfamethoxazole). All CF subjects were clinically stable during the time of the study and had an NIH clinical score (27) between 50 and 90. Control subjects were volunteers without history or symptoms of malabsorption and no other known clinical illness. The studies were approved by the institute's clinical research subpanel, and informed consent was obtained from all subjects (and parents, where applicable).

Dietary lipid supplements. The supplements used for the linoleic acid absorption test were commercial

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safflower oil (Hain Pure Food Co., Los Angeles, California; with 74% of fatty acids as 18:2ω6), Microlipid, a 50% emulsion of safflower oil (Organon, West Orange, New Jersey; with 74% of fatty acids as 18:2ω6); and Captex 810B and Captex 810D (Capitol City Products Co., Columbus, Ohio; with 25% and 40% of fatty acids as 18:2ω6, respectively). Captex 810B and 810D are experimental oils made of synthetic triglyceride molecules that contain both medium chain and long chain fatty acids randomly esterified to glycerol. These products are made by mixing fractionated coconut oil (medium chain triglyceride [MCT]) with sunflower oil in specific proportions, hydrolyzing all of the triglycerides and allowing random reesterification of the fatty acids present into composite triglyceride molecules that contain fatty acids from both of the starting oils (28–30). The linoleic acid content of the final products was determined by varying the proportions of the starting MCT and sunflower oils. Some patients were not able to participate in the full study because of the length of the hospital stay involved. When patients were unable to participate in the full study, every effort was made to insure that the individuals were given safflower oil, Microlipid and Captex 810D in a random order as mentioned below. Captex 810B was omitted since it differed from the Captex 810D only in linoleic acid content.

Test procedure. The different lipid supplements were provided to the CF patients and normal subjects in a random order. Fasting subjects consumed 36 g of lipid mixed in 300 ml of a modified Lundh test meal (31) composed of an instant breakfast drink (Carnation Instant Breakfast) containing 15 g of protein, 45 g of carbohydrate, <0.5 g of fat and <0.1 g of linoleic acid, mixed with a fat-free skim milk. Since Microlipid was a 50% emulsion, it was necessary to add 72 g of this product to obtain the 36 g of lipid dose. CF patients took their usual dosage of oral pancreatic enzymes with the meal. Blood was obtained from all subjects at 0, 2, 4, 6 and 8 hr after receiving the meal and was placed on ice. Blood samples were centrifuged to obtain plasma, which was transferred to a Teflon-lined screw-top culture tube, and stored frozen at −20 C under nitrogen until analysis. Absorption tests with different lipid preparations on any individual patient or control subject were performed at least three days apart. Both CF and control subjects were given a fat-free meal after their blood had been withdrawn at the 6-hr time point.

Analytical methods. Duplicate samples of 1 ml plasma were added to an equal volume of ethanol containing 50 mg of ascorbic acid as an antioxidant and saponified with potassium hydroxide, under nitrogen at 65–70 C, neutralized with concentrated hydrochloric acid to release fatty acids, and extracted two times with hexane. Hexane extracts were transferred quantitatively to preweighed beakers, the hexane was evaporated, and the beakers were desiccated for 2 hr prior to reweighing to determine the total fatty acids gravimetrically. Samples were extracted, and fatty acid methyl esters were prepared with boron trifluoride as previously described (32). Fatty acid methyl esters were resuspended in a small volume (50 μl) of isooctane, stored under nitrogen at 4 C, and analyzed by gas chromatography within 24 hr of methylation. Fatty acid methyl esters were separated on an Ultrapak 15% silar 10C on gas chrom R column (Applied Science, Deerfield, Illinois), temperature programmed from 185 C at 6 C/min to a final temperature of 225 C on a Finnigan 9500 (Finnigan, San Jose, California) gas chromatograph (GC) or a Hewlett-Packard 3800 GC (Hewlett-Packard, Palo Alto, California) with an HP 3380A integrator. Individual fatty acids were identified by comparison of retention times to pure standards (Supelco, Bellefonte, Pennsylvania). Levels of linoleic acid were expressed as area percent of the total amount of fatty acids present. Results were expressed as increase in area percent of linoleic acid or increase in mg/dl of total plasma fatty acids, compared to zero time levels. Results were analyzed for significance using Student's t-test (33).

RESULTS

All subjects demonstrated the ability to absorb the fatty acids contained in the various lipid supplements. However, there was a tendency to have greater variability in absorption, both for linoleic acid and total fatty acids, among the CF patients.

The change in total plasma fatty acids in CF patients and control subjects following the administration of the liquid meal containing safflower oil is shown in Figure 1. The increase of total plasma fatty acids following the administration of safflower oil in both control subjects and CF patients was essentially identical, suggesting equal absorption rates for this oil. In control subjects, the increase in area percent of plasma linoleic acid shows a similar pattern to that of the increase in total plasma fatty acids (Fig. 2). However, there was an apparent delay in the increase in area percent of plasma linoleic acid compared to the increase in total plasma fatty acids in CF patients after ingestion of the safflower oil test meals (Fig. 3). The mean peak increase observed in area percent of plasma linoleic acid level in CF patients was not significantly different from that of controls (9.36% vs 10.95%, respectively). The absorption patterns for total plasma fatty acids and area percent of plasma linoleic acid levels following administration of Microlipid (data not shown) in both CF and control subjects were similar to that seen with unemulsified safflower oil.

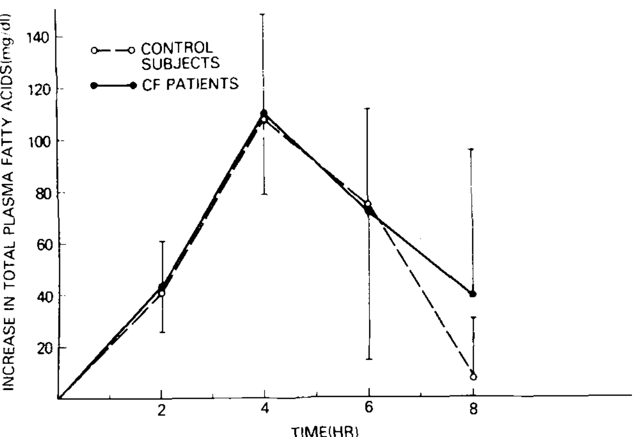

FIG. 1. Mean ± SEM increase in total plasma fatty acids over zero time values in control subjects (n = 7, ○) and cystic fibrosis patients (n = 9, ●) after ingestion of the 36-g dose of safflower oil in a test meal. Total plasma fatty acids were determined as described in Materials and Methods.

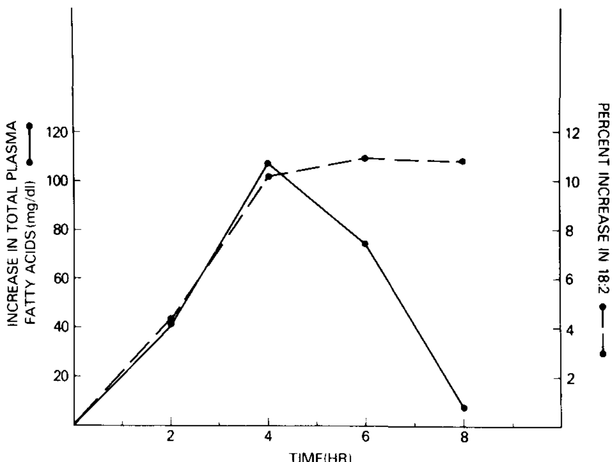

FIG. 2. Mean increase in total plasma fatty acids (solid line) and mean area percent increase of plasma linoleic acid (18:2) levels (dashed line) over zero time values in control subjects (n = 7) after ingestion of safflower oil. Plasma linoleic acid was determined as described in Materials and Methods.

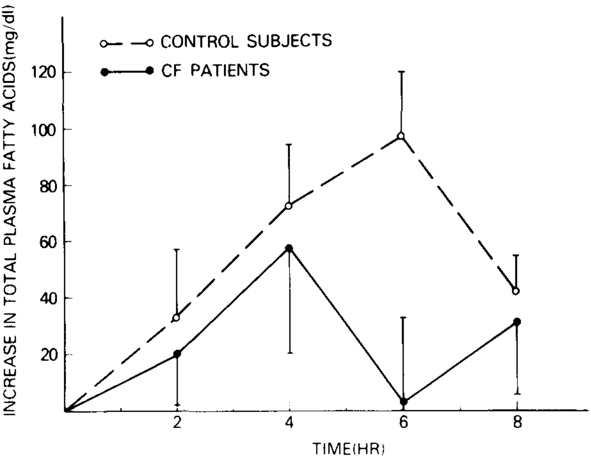

FIG. 4. Mean ± SEM increase in total plasma fatty acids over zero time values in control subjects (n = 7, ○) and cystic fibrosis patients (n = 6, ●) after ingestion of the 36-g dose of Captex 810D in a test meal.

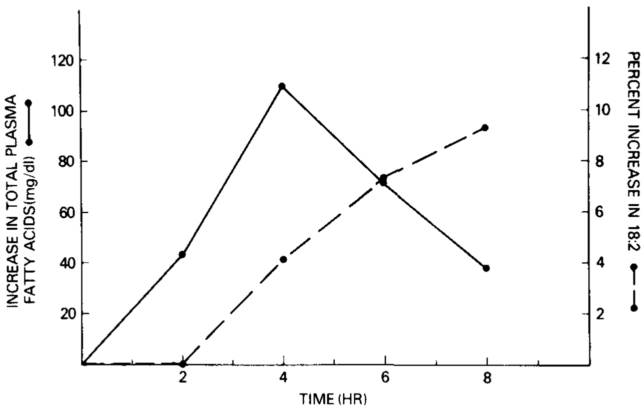

FIG. 3. Mean increase in total plasma fatty acids (solid line) and mean area percent increase of plasma linoleic acid (18:2) levels (dashed line) over zero time values in cystic fibrosis patients (n = 9) after ingestion of safflower oil. Note the delayed increase in percent linoleic acid relative to the increase of total plasma fatty acids.

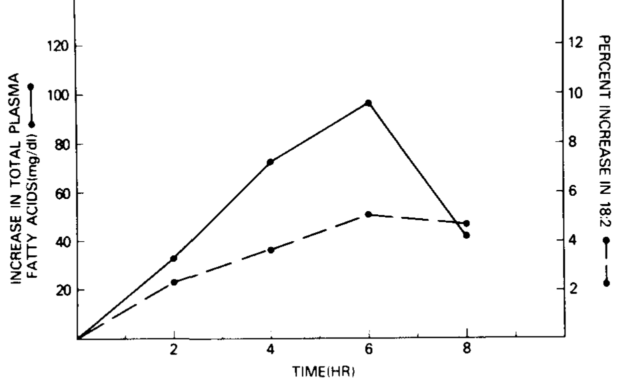

FIG. 5. Mean increase in total plasma fatty acids (solid line) and mean area percent increase of plasma linoleic acid (18:2) levels (dashed line) over zero time values in control subjects (n = 7) after ingestion of Captex 810D.

The pattern of increase in total plasma fatty acids following administration of Captex 810D also was similar for both CF and control subjects (Fig. 4). There appears to be a slight tendency for the CF patients to show a smaller increase in total plasma fatty acids relative to control subjects; however, no significant differences were found. In the control subjects, the increase in area percent of plasma linoleic acid occurred concurrently with the increase in total plasma fatty acids (Fig. 5), as it had with the safflower oil. However, the magnitude of change in area percent of plasma linoleic acid was less than that observed with the safflower oil preparation, since linoleic acid accounted for only 40% of the total fatty acids present in Captex 810D compared to 74% of the total fatty acids present in safflower oil. When Captex 810D was given to CF patients, the increase in area percent of linoleic acid occurred without a delay (Fig. 6), in contrast to the delayed increase following the ingestion of safflower oil (Fig. 3). In fact, with Captex 810D the peak

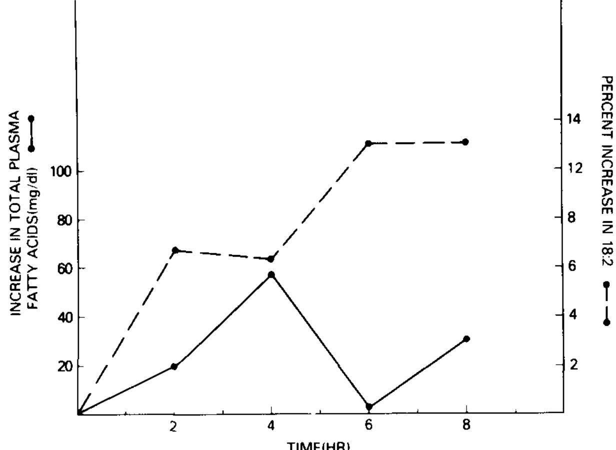

FIG. 6. Mean increase in total plasma fatty acids (solid line) and mean area percent increase of plasma linoleic acid (18:2) levels (dashed line) over zero time values in cystic fibrosis patients (n = 6) after ingestion of Captex 810D. Note the rapid rise in percent increase in 18:2 relative to the slower increase in total plasma fatty acids.

increase in area percent of plasma linoleic acid in CF patients was greater than the corresponding increase observed in control subjects ($p < 0.02$). When Captex 810B was given, the patterns of increase in total plasma fatty acids and area percent of plasma linoleic acid (data not shown) were similar to those observed with Captex 810D. However, the magnitude of the changes in area percent of plasma linoleic acid was smaller since linoleic acid accounted for only 25% of the total fatty acid content in Captex 810B.

DISCUSSION

The problem of marginal or inadequate linoleic acid status in CF patients has practical importance. Although altered fatty acid composition of blood and tissue lipids in the majority of CF patients may not be related to the basic defect, which is still unknown, there are data that suggest that altered lipid composition may potentiate some of the clinical manifestations of the disease (5,18,34).

Several types of lipid supplementation have been attempted in CF patients in an effort to correct the marginal essential fatty acid deficiency as well as to increase caloric intake (3,7,21–26). When MCT have been provided as the primary source of fat in the diet, alterations of fatty acid composition observed in lipids from CF patients became more abnormal (2). Long-term oral supplementation trials using corn oil or safflower oil have shown minimal or no improvement of linoleic acid status of CF patients (7,22,23). A problem with absorption of these oils has been proposed as one of the reasons for the failure to normalize linoleic acid status in these patients. However, it also has been suggested that underconsumption of calories related to their caloric needs (11,13) contributes to the marginal essential fatty acid status in CF patients. In this situation, the subject would utilize any ingested linoleic acid as a fuel for immediate energy needs rather than to meet essential fatty acid requirements.

Our data should help clarify the etiology of the abnormalities of fatty acid composition in CF patients. First, we have demonstrated that the magnitude of the increase in area percent of plasma linoleic acid and in total plasma fatty acids after ingestion of safflower oil is similar for both control subjects and CF patients as long as the CF patients are taking exogenous pancreatic enzymes at the time of the meal. Therefore, lack of absorption of the lipid supplement does not appear to be an adequate explanation of the altered fatty acid composition found in CF patients or of the lack of response to lipid supplementation trials. Second, the fact that the pattern of increase in total plasma fatty acids and corresponding increase in area percent of plasma linoleic acid after ingestion of the different lipid supplements investigated is different in the CF and control subjects supports the concept that the metabolism of the available dietary lipids is not the same in CF and control subjects.

In our previous work (13), it was not known whether the delay (relative to control subjects) in the increase in area percent of plasma linoleic acid observed in CF patients given the safflower oil test meal (Fig. 3) was due to differences in absorption of the ingested oil or a difference in metabolism of the absorbed products. However, the nearly identical pattern of change for total plasma fatty acids following the safflower oil test meal (Fig. 1) observed for both CF and control subjects suggests that a difference in absorption of the safflower oil in the two groups of subjects was unlikely. Thus, the delayed increase in area percent of plasma linoleic acid may be a consequence of increased metabolism of this unsaturated fatty acid in the relatively undernourished CF patients. CF patients with abnormal lipid fatty acid compositions also can be considered fat-deficient relative to control subjects. Consequently, our interpretation of the data is consistent with earlier studies in animals that demonstrated an increased rate of metabolism of orally fed linoleic acid to carbon dioxide in fat-deficient compared to normal mice (35).

The results observed following the administration of Captex 810D (Figs. 4–6) also support our hypothesis of selectively increased metabolism of certain fatty acids from the ingested lipids in the relatively malnourished CF patients compared to control subjects. Medium chain length fatty acids in the structured-lipid preparations provide a more preferable substrate for oxidative metabolism than long chain fatty acids (30,36,37). Thus, if these medium chain fatty acids are being more rapidly metabolized, the absorbed linoleic acid would appear as a larger proportion of the remaining plasma fatty acid pool.

The possibility of a more rapid absorption of Captex 810D compared to safflower oil in CF patients can be supported by the contribution of nonpancreatic lipases to the overall lipid digestion in these subjects (38). The preduodenal lipases are responsible for hydrolysis of dietary fat in the stomach. In earlier investigations with CF patients (38), this nonpancreatic lipolytic activity was attributed to lingual lipase, an enzyme secreted by the glands on the posterior aspects of the tongue. However, more recent studies have indicated that there is a gastric lipase that also contributes to intragastric lipolysis (39, 40). These preduodenal lipases have similar physical characteristics, including a low pH optimum and lack of dependence upon bile salts and co-lipase activity, that differ markedly from those characteristics of pancreatic lipase. The low duodenal pH (pH 4–5) and the relatively decreased levels of intraluminal bile salts in CF patients allow the persistence of lipolytic activity of these enzymes in the upper small intestine of CF subjects (38,40–42). Lingual lipase and presumably gastric lipase act preferentially on shorter chain triglycerides (43) such as those present in the Captex products, which would favor the utilization of these products compared to those containing long chain triglycerides such as safflower oil.

The data presented suggest that the structured-lipid preparations investigated provide efficient vehicles for the supplementation of linoleic acid to the CF patient. Our results indicate that malabsorption alone cannot account for the inadequate or marginal essential fatty acid status of CF patients, and that long-term consumption of supplemental linoleic acid in addition to adequate caloric intake should improve the linoleic acid status of most, if not all, CF patients. The interpretation of our data is consistent with observations reported by others (24–26). Randomly esterified synthetic triglycerides such as the structured lipids used in this study offer the advantage of providing shorter chain length fatty acids as a source of immediate energy through oxidative metabolism at the same time as providing essential fatty acids for repletion and maintenance of tissue stores. Such products would

seem especially well-suited to CF patients since the presence of shorter chain length fatty acids appears to enhance their utilization in this population of subjects.

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Medium Chain Triglyceride in Early Life: Effects on Growth of Adipose Tissue[1]

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Effects of feeding early in life a diet high in either long chain (LCT) or medium chain triglyceride (MCT) were studied on the development of adipose tissue in postweanling rats. The diets were similar in calorie distribution and identical in nutrients except for type of fat. The caloric distribution of the two diets by percent was LCT (corn oil)/protein/carbohydrate, 70/18/12 and MCT/corn oil/protein/carbohydrate, 66/4/18/12. Male littermates with less than 5% weight difference were pair-fed the two diets randomly at age 18–20 days. One-fourth of the rats were killed at 10, 16, 22 and 28 weeks of age and analyzed for adipose depots and adipose tissue cellularity. Results showed that the LCT-fed rats were significantly heavier, with larger epididymal, retroperitoneal, omental and subcutaneous fat pads than the respective pair-fed MCT rats. Also, LCT-fed rats had larger size and number of adipocytes than MCT-fed littermates. It is concluded that the type of fat in the diet, namely LCT or MCT, when fed early in life can influence the development of adipose tissue. MCT appears less lipogenic than LCT. The mechanism for the diminished adiposity of MCT-fed rats is related to extensive oxidation of MCT and its enhancement of thermogenesis leading to lessened energy efficiency.
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Tailormade medium chain triglycerides (MCT) for human consumption have been investigated and in use for over 25 years. Several reviews have dealt with their absorption, transport, metabolism and clinical uses (1–3). In the United States, the available MCT preparations are triglycerides whose constituent medium chain fatty acids (MCFA) are octanoic acid, 8:0 (65–70%); decanoic acid, 10:0 (20–35%); hexanoic acid, 6:0 (1–2%); and dodecanoic acid, 12:0 (1–2%). In Europe, MCT preparations tend to contain lower proportions of 8:0 and higher proportions of 10:0, at times in equal amounts, with trace quantities of 6:0 and 12:0. Although MCFA do appear in naturally occurring mixed triglycerides, pure edible MCT are derived semisynthetically from coconut oil or milk fat. For example, the fatty acids of coconut oil are hydrolyzed and molecularly distilled to yield three fractions: MCFA, relatively pure 12:0 and long chain fatty acids. MCFA are purified prior to reesterification with glycerol to form MCT (4). The final product (MCT) is a clear, light yellow oil with a melting point of −5 C, made up entirely of saturated MCFA ranging in length from 6:0 to 12:0.

Recently, studies of digestion, absorption and transport of MCT have been reviewed (3). It is clear that MCT is hydrolyzed efficiently in the lumen of the small intestine under conditions adverse to the hydrolysis of LCT (Fig. 1). Unlike LCT, in the absence of pancreatic lipase MCT is absorbed into the mucosa of the intestine, where it is hydrolyzed by a mucosal lipase into MCFA, which traverse the capillaries and are transported via the portal vein into the liver. In the liver, MCFA are extensively oxidized and, for this reason, are not incorporated into lipid ester moieties of lipoproteins. Also, less than 3% of ingested MCT are transported as chylomicrons. In view of the efficiency of their absorption and the uniqueness of their mode of transport, MCT have been used in the treatment of a variety of malabsorption syndromes, including long chain fat malabsorption in premature infants (5–7).

Infants and adults fed MCT as a major proportion of calories do not incorporate MCFA into adipose tissue. Feeding MCT to premature infants as 80% of dietary fat in formula diets deriving 50% of calories from fat has been shown to improve fat absorption and nitrogen retention (5,6). The growth curve of the MCT-fed infants did not differ significantly from that of the LCT-fed infants. However, the MCT-fed infants appeared more lean and

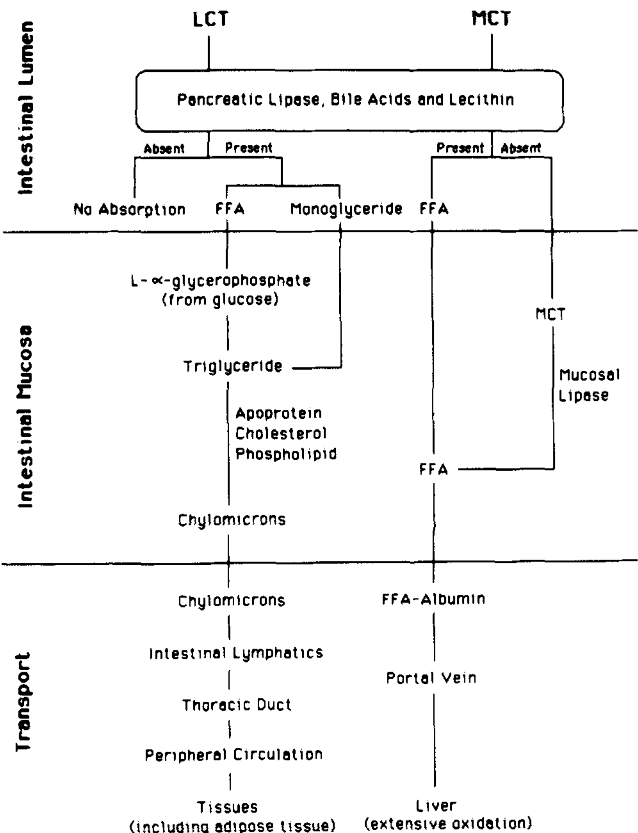

FIG. 1. Digestion, absorption and transport of long chain (LCT) and medium chain triglycerides (MCT). FFA, free fatty acid.

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less fat clinically than the LCT-fed infants. Unfortunately, studies of body composition were not done on these infants. However, it was possible to study the effect of MCT on fat deposition in the rat (8). Three groups of rats were fed either a low fat diet, a diet containing 55% (by energy) MCT and 5% corn oil, or a diet containing 60% LCT in the form of corn oil. The results showed that, unlike LCT, MCT had a reductive effect on fat depots and, like LCT, had a depressive effect on lipogenesis. In contrast, the low fat, high carbohydrate diet had an enhancing effect on lipogenesis. In another study (9) in which food intake was precisely controlled by gastrostomy, body composition was measured in rats overfed with MCT- or LCT-containing diets that provided 50% calories in excess of normal consumption. The MCT-fed rats showed significantly smaller fat depots and adipocyte size than the LCT-fed controls. In a similar study, the mechanism of the reductive effect of MCT on fat depots was related in part to increased metabolic rate and enhanced thermogenesis (10).

The mass of adipose tissue in man and animals is dependent upon the number and size of its adipocytes. It is therefore conceivable to modify the ultimate dimension of the adipose depot in the body by factors that affect adipocyte replication and/or adipocyte size. In man and rat, the number of adipocytes has been reported as fixed at a certain age (11-13). During the postnatal period of growth, adipocyte proliferation continues until a maximum is attained, whereas further growth of adipose tissue occurs primarily as a result of adipocyte enlargement and not cell division (11). However, it has been reported that the adipocyte number can be increased in adult rats by feeding a diet high in long chain fat (14). Similarly, in the Osborne-Mendel rat, a high fat diet markedly increased the adipose tissue mass without significantly altering water, protein or ash content (15). The average human newborn infant has 500 g of fat in 4 billion adipocytes, each containing 0.12 μg of fat (16). In contrast, the normal nonobese adult has 30 billion adipocytes with 0.5 μg of fat per cell, while the obese adult may harbor 1 trillion adipocytes, each containing 0.6 to 1.2 μg of fat (17). Thus, a considerable increase in adipocyte number must occur from infancy to adulthood. Little is known about the regulatory mechanisms that control the growth of adipose tissue. Nutrition appears to be important. Preweaning undernutrition has been shown to result in an ultimate reduction in the cell number of the epididymal fat body of the rat (18). While undernutrition can result in a reduction of adipocyte number (and size), such a manipulation also can produce appreciable reduction in the cell number of the central nervous system (19). It is postulated that MCT feeding in early life will reduce adipose tissue mass with concurrent preservation of adequate nutrition. It is also postulated that chylomicrons resulting from the ingestion of LCT and not MCT are important stimuli for growth of adipose tissue. As LCT digestion and absorption improve postnatally, there is rapid accumulation of fat, associated with enhanced cellularity of the adipose tissue.

MATERIALS AND METHODS

Pregnant Sprague-Dawley rats fed a standard stock diet were allowed to deliver and nurse their pups in our laboratory. At weaning (18-20 days), male littermates from the same dam with weight difference of 5% or less were pair-fed and divided into two groups, to be fed an LCT or MCT diet. MCT was provided by Capital City Products Co. (Columbus, Ohio). The diets were identical in nutrients except for the type of fat and were similar in energy distribution. The LCT diet derived 70% of total energy from corn oil; the MCT derived 66% of total energy from MCT and 4% from corn oil (to supply essential fatty acid). Both diets contained 18% of total energy as protein and 12% as sucrose. Each diet yielded 3.9 kcal/g. The composition of the diets is shown in Table 1. Diet was placed in a cup anchored to the cage and covered with a screen mass and a broad funnel-like lid. In this way spillage was negligible. The animals were pair-fed their respective diets from weaning until they were killed. The animal that ate the least (usually the MCT rat) was allowed to eat ad libitum. The exact amount of food eaten by this rat was then given to the other rat of the pair at the next feeding. The amount of diet consumed was weighed and calculated three times per week. One-fourth of the rats were killed at 10, 12, 22 and 28 weeks of age. Various fat pads were removed as follows: A ventral midline vertical incision was cut toward the head. Two cuts were made laterally from the midline incision about 1 cm above the hip. Each epididymal pad was exposed and cut at the base of the epididymis toward the epididymal vessels. The cut then followed the vessels until the whole epididymal pad was freed. The left epididymal pad was used to determine number and size of the adipocytes. The retroperitoneal fat was removed by cutting its apex

TABLE 1

Composition of Diets Containing Either Long Chain (LCT) or Medium Chain Triglyceride (MCT)

	LCT (wt %)	MCT (wt %)
Casein	17.6	17.6
Sucrose	11.4	11.4
Corn oil	30.4	1.7
MCT		31.1
Alphacel	35.8	33.4
Salt mixture[a]	3.8	3.8
Vitamin mixture[b]	1.0	1.0
Caloric density (kcal/g)	3.9	3.9
Caloric distribution (%)		
Protein	18	18
Fat	70	70
Carbohydrate	12	12

[a]USP XIV salt mixture from ICN Nutritional Biochemicals. Contains (g/100 g mixture): cupric sulfate, 0.007; ferric ammonium citrate, 1.53; manganese sulfate, 0.02; ammonium alum, 0.009; potassium iodide, 0.004; sodium fluoride, 0.051; calcium carbonate, 6.86; calcium citrate, 30.83; calcium biphosphate, 11.28; magnesium carbonate, 3.52; magnesium sulfate, 3.83; potassium chloride, 12.47; potassium phosphate diabasic, 21.88; sodium chloride, 7.71; zinc carbonate, 0.0024.

[b]Vitamin diet fortification mixture from ICN Nutritional Biochemicals. Contains (g/100 g mixture): retinyl acetate (200,000 units/g), 2.95; cholecalciferol (400,000 units/g), 0.16; tocopherol, 3.30; choline chloride, 49.20; menadione, 1.47; p-aminobenzoic acid, 3.18; niacin, 3.18; riboflavin, 0.66; pyridoxine hydrochloride, 0.66; thiamin hydrochloride, 0.66; calcium pantothenate, 1.97; biotin, 13.1 mg; folic acid, 59.0 mg; vitamin B_{12}, 0.9 mg.

at about the midinguinal point and at the medial border of the psoas muscle. The cut then followed the medial border of the psoas muscle until it reached just below the lower pole of the kidney and followed the lateral border of the kidney as far as possible toward the diaphragm. The retroperitoneal fat was freed from the underlying muscle and weighed. The left retroperitoneal fat was used to determine number and size of the adipocytes. The dorsal fat is a rectangular pad. The vertical lines ran through the shoulder from a point 1 cm above the hip to the neck. The caudal horizontal line ran from one vertical line to the other, 1 cm above the hip. The skin was carefully dissected out free of fat. Then this dorsal fat pad was carefully removed free of muscle and brown fat. It was weighed and used to determine cell number and size. The remaining subcutaneous fat was then dissected out and weighed; the omental fat was stripped off easily from the alimentary tract. Thus, the total fat was comprised of all the fat pads: right and left epididymal fat, right and left retroperitoneal fat, dorsal fat and remaining subcutaneous fat.

The cell count was determined by the method of Hirsch and Gallian (20) as follows: A certain amount of adipose tissue (one sample each from the left epididymal and retroperitoneal and two from the dorsal fat) weighing about 80 mg in several small pieces from different sites was weighed on a tared nylon mesh (200 μ) and placed in a polyethylene vial containing 15 ml of 2% osmium tetroxide in 0.05 M collidin buffer, pH 7.4. The tissue was incubated at 37 C for 48 hr. It was thoroughly washed, and the freed adipocytes were counted in a Coulter counter. The total adipocytes per piece of adipose tissue of known weight were thus calculated.

The cell size was determined as follows: One piece each of left epididymal, left retroperitoneal and dorsal fat (about 150 mg) were placed in separate 50-ml ground-glass stoppered tubes, followed by 20 ml of redistilled chloroform and 10 ml of redistilled methanol. The tube was allowed to stand in a cold room for 48 hr. Distilled water was added to the tube, which was shaken gently and allowed to stand for separation of the solution in the tube into two phases. The upper aqueous phase was removed. About 10-15 g of anhydrous sodium sulfate was added to the chloroform phase and shaken gently. Then 5 ml of the lipid phase was pipetted into a tared dish. The chloroform was allowed to evaporate completely, and the remaining lipid in the dish was weighed. Thus, the lipid content of the adipose tissue of known weight was divided by the number of cells, and in this way converted into cell size, expressed as μg of lipid (mainly triglyceride) per cell.

RESULTS

Body weight and weights of both epididymal, both retroperitoneal, total subcutaneous and omental fat pads of the two groups of rats at ages 10, 16, 22 and 28 wk are shown in Table 2. The LCT-fed rats were substantially heavier than their pair-fed MCT counterparts at all ages. The increment in body weight in the LCT over the MCT rats increases with age from 3.7% at age 10 wk to 8.5% at age 16 wk to 15% at ages 22 and 28 wk. All fat pads in the LCT group were significantly heavier than those of the pair-fed MCT rats at all ages. The increases in the weights of the epididymal, retroperitoneal and omental fat pads in the LCT group over those of the MCT group were 30-35% at age 10 wk and approximately 50% at ages 16, 22 and 28 wk. The subcutaneous fat, the largest dissectible fat pad in the rat, was 18-30% heavier in the LCT than in the MCT group at all ages. The total dissectible fat without the omental pad (so computed because the weights for the omental fat pads were not obtained at 22 and 28 wi) was 27-36% heavier in the LCT than in the MCT group at all ages.

The total lipid content of the left epididymal, left retroperitoneal and interscapular (dorsal) subcutaneous fat pads of the LCT and MCT groups at ages 10, 16, 22 and

TABLE 2

Body Weight and Weights of Various Fat Pads of Rats Pair-Fed Diets of Either 70% LCT or 70% MCT at Ages 10, 16, 22 and 28 Wk

	10 Wk		16 Wk		22 Wk		28 Wk	
	LCT	MCT	LCT	MCT	LCT	MCT	LCT	MCT
No. of rats	8	8	8	8	8	8	9	9
Body weight	269 ± 13[a]	259 ± 11	390 ± 15[c]	357 ± 14	472 ± 21[c]	407 ± 18	480 ± 15[d]	418 ± 14
Total epididymal pads	3.17[b] ± 0.43	2.10 ± 0.19	5.95[d] ± 0.46	3.12 ± 0.30	7.24[c] ± 0.89	3.87 ± 0.42	6.72[d] ± 0.48	3.69 ± 0.37
Total retroperitoneal pads	3.79[c] ± 0.55	2.47 ± 0.40	9.82[c] ± 1.05	4.57 ± 0.61	12.44[c] ± 1.92	5.91 ± 0.79	11.64[c] ± 1.77	5.58 ± 0.85
Subcutaneous pad	22.27[a] ± 2.81	16.86 ± 1.44	35.14[c] ± 2.43	24.74 ± 1.81	41.83[b] ± 4.45	30.57 ± 2.60	37.25[c] ± 3.06	30.41 ± 2.78
Omental pad	5.27[c] ± 0.55	3.78 ± 0.36	10.42[d] ± 0.59	5.32 ± 0.42				
Total fat without omental pad	29.24[a] ± 3.73	21.43 ± 1.98	50.50[d] ± 3.73	32.45 ± 2.48	61.51[c] ± 6.99	40.35 ± 3.71	55.61[d] ± 5.04	38.68 ± 3.93

Values represent mean ± SE, g.

[a]Significantly different from MCT rats at the same age: $p < .025$.

[b]Significantly different from MCT rats at the same age: $p < .01$.

[c]Significantly different from MCT rats at the same age: $p < .005$.

[d]Significantly different from MCT rats at the same age: $p < .001$.

28 wk is shown in Table 3. The LCT rats had significantly more lipid per unit weight of the three fat pads than their respective pair-fed MCT rats, except for the dorsal pad at 28 wk.

The fat cell size of the left epididymal, retroperitoneal and interscapular (dorsal) subcutaneous fat pads of the two groups at all ages is shown in Table 4. The LCT rats had significantly larger adipocytes in all depot fat pads than the MCT rats at all ages, except for the epididymal fat pad at age 10 wk and the dorsal fat pad at 28 wk.

The fat cell number of the left epididymal, retroperitoneal and interscapular (dorsal) subcutaneous fat pads of the two groups of rats at all ages is shown in Table 5. For the epididymal fat pad, the LCT rats tended to have a larger adipocyte number than the MCT rats at all ages, but the difference was significant only at 28 wk. For the retroperitoneal fat pad, the LCT rats had significantly more adipocytes at all ages. In contrast, the number of fat cells in the dorsal pad did not differ significantly between the LCT and the MCT groups.

DISCUSSION

The data show that the LCT-fed rats were significantly heavier than the respective pair-fed MCT rats at all ages. Also, the various adipose depots weighed on an average 30% to 50% more in the LCT- than in the MCT-fed rats. It is noteworthy that both groups of animals consumed the same amount of calories, and their diets, except for quality of fat content, were identical in caloric distribution and density. Under the conditions of pair-feeding, rats in the LCT group tended to finish their food, while those in the MCT group usually had food available throughout the 24 hr. Thus, the LCT-fed rats tended to be hungry and therefore more active than the MCT-fed rats. If the activity of both groups had been similar, the LCT-fed rats would have been heavier and their adipose depots larger, and the difference in the parameters between the two groups would have been more pronounced. A possible explanation for the diminished spontaneous food intake of rats fed MCT in comparison with animals

TABLE 3

Total Lipid Content of Left Epididymal, Left Retroperitoneal and Dorsal Fat Pads of Rats Pair-Fed Diets of Either 70% LCT or 70% MCT at Ages 10, 16, 22 and 28 Wk

Age (wk)	No. of rats	Left epididymal pad		Left retroperitoneal pad		Dorsal pad	
		LCT	MCT	LCT	MCT	LCT	MCT
10	8,8	$84.4^{e} \pm 1.4$	79.6 ± 0.9	$82.2^{c} \pm 3.1$	77.1 ± 2.9	$79.1^{b} \pm 3.2$	69.8 ± 1.0
16	8,8	$89.5^{e} \pm 0.8$	83.1 ± 1.1	$90.0^{e} \pm 0.6$	83.2 ± 0.8	$84.1^{d} \pm 1.2$	70.2 ± 1.9
22	8,8	$88.2^{d} \pm 1.3$	81.1 ± 0.7	$86.4^{a} \pm 1.6$	81.9 ± 0.9	$77.5^{e} \pm 1.6$	68.8 ± 1.3
28	9,9	$83.9^{d} \pm 0.4$	70.9 ± 0.9	$85.2^{d} \pm 0.9$	81.0 ± 1.2	74.2 ± 1.0	71.2 ± 1.6

Values represent mean ± SE, % w/w.
[a]Significantly different from MCT rats at the same age: $p < .05$.
[b]Significantly different from MCT rats at the same age: $p < .025$.
[c]Significantly different from MCT rats at the same age: $p < .01$.
[d]Significantly different from MCT rats at the same age: $p < .005$.
[e]Significantly different from MCT rats at the same age: $p < .001$.

TABLE 4

Fat Cell Size of Left Epididymal, Left Retroperitoneal and Dorsal Fat Pads of Rats Pair-Fed Diets of Either 70% LCT or 70% MCT at Ages 10, 16, 22 and 28 Wk

Age (wk)	No. of rats	Left epididymal pad		Left retroperitoneal pad		Dorsal pad	
		LCT	MCT	LCT	MCT	LCT	MCT
10	8,8	0.285 ± 0.030	0.221 ± 0.022	$0.384^{a} \pm 0.048$	0.298 ± 0.033	$0.229^{a} \pm 0.028$	0.158 ± 0.010
16	8,8	$0.656^{b} \pm 0.066$	0.381 ± 0.081	$0.720^{c} \pm 0.042$	0.391 ± 0.056	$0.342^{d} \pm 0.014$	0.188 ± 0.017
22	8,8	$0.481^{d} \pm 0.024$	0.280 ± 0.026	$0.670^{c} \pm 0.055$	0.429 ± 0.036	$0.297^{b} \pm 0.022$	0.188 ± 0.014
28	9,9	$0.435^{c} \pm 0.024$	0.285 ± 0.033	$0.672^{d} \pm 0.046$	0.429 ± 0.041	0.285 ± 0.022	0.231 ± 0.040

Values represent mean ± SE, μg lipid per fat cell.
[a]Significantly different from MCT rats at the same age: $p < .05$.
[b]Significantly different from MCT rats at the same age: $p < .025$.
[c]Significantly different from MCT rats at the same age: $p < .005$.
[d]Significantly different from MCT rats at the same age: $p < .001$.

TABLE 5

Fat Cell Number of Left Epididymal, Left Retroperitoneal and Dorsal Fat Pads of Rats Pair-Fed Diets of Either 70% LCT or 70% MCT at Ages 10, 16, 22 and 28 Wk

Age (wk)	No. of rats	Left epididymal pad		Left retroperitoneal pad		Dorsal pad	
		LCT	MCT	LCT	MCT	LCT	MCT
10	8,8	4.821 ± 0.488	3.892 ± 0.306	4.266[d] ± 0.342	3.204 ± 0.371	8.589 ± 0.703	6.598 ± 0.467
16	8,8	4.372 ± 0.566	4.203 ± 0.618	6.231 ± 0.510	5.244 ± 0.526	9.617 ± 0.676	10.898 ± 1.248
22	8,8	6.306 ± 0.534	5.746 ± 0.308	7.613[b] ± 0.946	5.465 ± 0.421	12.620 ± 1.676	12.081 ± 1.144
28	9,9	6.534[a] ± 0.386	5.366 ± 0.374	7.581[c] ± 0.793	5.264 ± 0.404	10.270 ± 0.609	10.606 ± 1.029

Values represent mean ± SE, × 10^6.
[a]Significantly different from MCT rats at the same age: $p < .05$.
[b]Significantly different from MCT rats at the same age: $p < .025$.
[c]Significantly different from MCT rats at the same age: $p < .01$.
[d]Significantly different from MCT rats at the same age: $p < .001$.

fed LCT ad libitum is the development of hyperketonemia during MCT feeding (2,21,22). For this reason, the present study used pair-feeding to ensure similarity in food intake in both groups of animals. Rats fed ad libitum a diet containing 20% lard were reported to be heavier than animals fed a diet containing 20% MCT and had larger epididymal fat pads. The energy requirement for weight maintenance was higher in rats fed MCT than in those fed lard (23). Pigs fed a 10% MCT diet exhibited weight gain and feed efficiency at a rate considerably lower than that of animals fed a 10% LCT diet (24). Premature infants fed a formula containing 40% of total calories from MCT gained less weight than infants fed an identical formula containing 50% of total energy from LCT (corn oil), despite the fact that the groups had the same calorie intake and that the MCT-fed infants absorbed more fat, protein, calcium and magnesium than the LCT-fed group (5,6).

This study supports the hypothesis that MCT feeding early in life results in diminished fat deposition and adipose tissue cellularity. The mechanism whereby MCT feeding results in diminished adiposity compared to LCT feeding may be related to vast differences in the modes of digestion, absorption and metabolism of the two triglycerides. In contrast to LCT, which appear in the systemic circulation as chylomicrons, the MCT-derived fatty acids are transported via the portal vein (25) to the liver, where they are extensively oxidized into CO_2 and other water-soluble metabolites, such as ketones, and are not incorporated into hepatic lipids to any appreciable extent (1,2). In the rat, the cumulative oxidation of labeled MCT over a period of 48 hr was about twice that of LCT (26). In both man and animals, the oxidation of MCT-derived fatty acids is not appreciably affected by the nutritional state. In contrast, feeding drastically reduces the oxidation of LCT-derived fatty acids (27). Thus, it appears that MCT must undergo obligatory oxidation in the liver after absorption and transport. The oxidative aqueous products other than CO_2 reach the systemic circulation and are utilized by various organs, including the adipose tissue. Theoretically the adipose tissue can synthesize long chain fatty acids from MCT-derived oxidative products. However, studies have shown that the adipose tissue in fact contains very little medium chain fatty acid (3). In contrast, the LCT moieties of chylomicrons are hydrolyzed, picked up and readily reesterified into triglycerides by the adipose tissue. Thus, the metabolic steps that lead to formation of triglycerides in adipose tissue are much simpler for LCT than for MCT. In the intact animal and in man, evidence suggests that MCT feeding is associated with enhanced thermogenesis, thus favoring diminished energy efficiency and reduced deposition of fat (10,28).

LCT-fed rats had not only larger fat depots but also higher lipid content per unit weight than MCT-fed rats. The weight increase of various fat depots in the LCT-fed rats was due to adipocytes being bigger and more numerous than in the MCT-fed rats. It is not known whether the increase in adipocyte number in the LCT group is due to actual proliferation or to fattening up of existing pre-adipocytes.

In virtually all mammalian species, the mammary gland synthesizes LCT and varying proportions of MCT (29). In rabbit milk, the predominant fat is MCT, reaching 80% of the milk fat. Smaller proportions of MCT in milk fat occur in the rat (30%), in the mouse (8%) and in man (12%). It appears that the mammary gland begins to synthesize MCT during pregnancy and maintains such synthesis during lactation through a remarkable mechanism involving an enzyme (thioesterase II) that segments long chain fatty acids (LCFA) into MCFA for incorporation into milk triglycerides (30). The fatty acid distribution on the triglyceride appears to favor digestion and absorption of the milk fat by the neonate in that the LCFA tend to occupy the 1 and 2 positions, while the 3 position is occupied by the MCFA (31,32). The net effect of this presentation of milk fat to the neonate is ease of hydrolysis and subsequent elaboration of MCFA, LCFA and long chain monoglycerides for enhanced intestinal absorption. Studies in man suggest a twofold increase in MCFA in breast milk of mothers at premature delivery and that the premature infant, when ingesting its own mother's milk for three months, receives 17% of total fat as MCFA, in contrast to 10% for the full-term infant (33). By increasing the proportion of dietary MCFA from MCT, it may be possible to circumvent the diminished

ability of the premature infant to digest and absorb LCT and concurrently to offer an easily oxidizable fat. The effect of starting MCT feeding in the neonatal period on the development of adipose tissue cellularity awaits further investigation. Our study demonstrates that the type of fat in the diet in the postweaning period can influence the development of adipose tissue cellularity. MCT feeding early in life may offer a potential measure for prevention and control of human obesity.

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The Effect of Medium and Long Chain Triglyceride on Human Adipose Tissue Metabolism[1]

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Studies have been performed to assess the effects, in vivo and in vitro, of lipid emulsions on human adipose tissue prostaglandin production. Subcutaneous adipose tissue obtained either during elective surgery or by needle aspiration was studied in tissue culture or by using a perifusion apparatus. Physical mixtures of emulsions of long chain triglyceride (LCT) and/or medium chain triglyceride (MCT) were added to the tissue culture medium so that the final concentration was 400 mg/dl. After a 3-day incubation period the tissue was harvested, placed in buffer and used to determine in vitro production of prostaglandin E_2, prostacyclin I_2 (measured as its stable end product 6-keto $PGF_{1\alpha}$) and thromboxane A_2 (measured as TXB_2) by radioimmunoassay. The results demonstrated that samples incubated in 100% MCT had the most significant increase in prostaglandin production, while those incubated in 100% LCT had the most significant decrease in activity of the three prostaglandins assayed. The addition of LCT to MCT caused a stepwise decrease in adipose tissue prostaglandin production. The data suggest a pharmacological rather than a physiological effect of lipid emulsions containing MCT and/or LCT on adipose tissue prostaglandin production. In vivo effects of a 20% safflower oil emulsion, containing high levels of the essential fatty acid linoleate, were assessed in five pediatric patients. Adipose tissue was obtained before and after two and four weeks of treatment. Fatty acid profiles and prostaglandin production were determined. The results demonstrated that intravenous fat infusion increased the concentrations of linoleic and arachidonic acids found in adipose tissue within a short interval. The effect of intravenous fat infusion on human adipose tissue prostaglandin production was less predictable and may have been a function of the patients' disease and subsequent clinical course. These findings suggest that lipid emulsions should not be viewed solely as a source of intravenous energy, because they may have the potential to elicit changes in prostaglandin production as demonstrated by a human adipose tissue model.
Lipids 22, 435–441 (1987).

Adipose tissue is a dynamic organ central in maintaining caloric balance; it is capable of responding to a variety of metabolic, hormonal and nutritional influences. The structure and function of adipose tissue have generally been studied in the context of overnutrition as seen in the obese. The possible role of adipose tissue in the recovering, malnourished, hospitalized patient has largely been ignored.

The 10% and 20% lipid emulsions have been widely used as a source of intravenous energy or as a means for preventing or ameliorating essential fatty acid (EFA) deficiency in malnourished patients. The emulsions currently used contain 54% to 77% linoleic acid, an EFA. Linoleic acid is the biosynthetic precursor for another EFA, arachidonic acid. Further modification of this fatty acid yields a family of bioactive metabolites, commonly called the eicosanoids. These products include the prostaglandins, leukotrienes and hydroperoxy-arachidonate derivatives. It has been hypothesized that the high levels of linoleic acid delivered as part of a total parenteral nutrition (TPN) regimen may affect endogenous prostaglandin production. It has been demonstrated that fat emulsions are principally removed from the bloodstream by skeletal muscle and adipose tissue (1) and that adipose tissue produces prostaglandins (2–5); it thus was of interest to determine the effect of this nutriment on fat tissue metabolism.

MATERIALS AND METHODS

Experiments in our laboratory have been performed to assess the effect in vivo and in vitro of various lipids, triglycerides and fatty acids on the ability of human adipose tissue to make prostaglandins. To perform these experiments, we modified the perifusion apparatus (PA) of Allen et al. (6) to study the tissue under more physiological conditions. The PA is a glass water-jacketed column in which fat tissue is suspended and kept at a constant 37 C. Krebs-Ringer bicarbonate buffer (pH 7.4) is pumped through the top at a constant rate and automatically collected at the bottom using a fraction collector. At various intervals, any combination of substrates or drugs can be infused by a series of pumps. During the experiments described below, two PA were run simultaneously. One column functioned as a control, i.e., only buffer was pumped through, while the other was used for the experimental manipulation. The advantage of this system compared to the more traditional tissue culture is that, with the constant turnover of buffer through the PA, there are no metabolic end products in the chamber that may affect the response of the tissue under study. In the first series of experiments, adipose tissue was obtained from four obese subjects by needle aspiration using the method of Hirsch and Goldrick (7). The tissue was washed in cold 0.9% sterile saline and split into equal portions of 200–300 mg. The tissue was then suspended in the two PA and allowed to equilibrate for 5 min. The buffer used contained fatty acid–free albumin (5 mg/dl) and glucose (100 mg/dl) and was pumped at a rate sufficient to clear the volume of the column every 5 min. After a baseline period of 20 min, arachidonic acid that had been previously bound to albumin (8) at a concentration of 0.3–0.5 μmol/ml was infused into the experimental column for the duration of the experiment. Fractions were collected every 5 min and analyzed by radioimmunoassay (RIA) for prostaglandin E_2 (PGE_2), prostacyclin I_2 (measured as the stable metabolite 6-keto $PGF_{1\alpha}$) and thromboxane A_2 (measured as the stable metabolite TXB_2) (9–14).

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In vitro effects of lipid emulsions on adipose tissue. In this investigation, physical mixtures of lipid emulsions containing 20% long chain triglyceride (LCT) from soybean oil and/or medium chain triglyceride (MCT) were made to assess the effect of concentrations of linoleic acid ranging from 0 to 54% (Katz, D.P., and Knittle, J.L., submitted for publication).

Subcutaneous human adipose tissue was obtained during elective abdominal surgery and resected from pieces that would otherwise have been discarded. All subsequent manipulations of the tissue were performed in a laminar flow hood. The tissue was washed in 0.9% sterile saline and then placed in tissue culture for 24 hr to equilibrate at 37 C in defined culture medium, RPMI 1640 with 20% fetal calf serum (Gibco Laboratories, Grand Island, New York). The defined culture medium with fetal calf serum contained less than 0.01% of the essential fatty acids, linoleic and arachidonic. At the end of the 24-hr period the tissue was harvested and washed in multiple volumes of 0.9% sterile saline. The tissue was then divided into portions of 200-300 mg each and placed into seven duplicate tissue culture flasks. To each flask 10 ml of the medium described above was added. At this time a physical mix of the different lipid emulsions to be tested was added to the duplicate flasks so that the final triglyceride concentration was 400 mg/dl. The flasks were then placed in a 37 C CO_2 incubator on a rocker platform (Bellco Glass, Vineland, New Jersey) to insure proper mixing. The tissue remained in culture for 3 days and was fed each day, as above, after the old medium was removed. At the end of the 3-day period the tissue was harvested and washed in 0.9% sterile saline. The samples were then used in experiments to measure in vitro prostaglandin production.

The tissue was separated into equal portions and placed in duplicate tissue culture flasks with Krebs-Ringer bicarbonate buffer (pH 7.4) containing fatty acid-free bovine serum albumin (5 mg/dl) (Sigma Chemical Co., St. Louis, Missouri) as prepared by the method of Chen (15) and glucose (1 mg/ml). It was then placed back into the CO_2 incubator on the rocker platform for 60 min. At the end of this incubation period the buffer was recovered and aliquoted for the determination of prostaglandins. The samples were analyzed in duplicate for prostacyclin I_2 (measured as the stable metabolite 6-keto $PGF_{1\alpha}$), thromboxane A_2 (measured as TXB_2) and PGE_2 by RIA (9-14). Determinations of fat cell size were made in each set of experiments so the data could be standardized (16).

In vivo effects of a lipid emulsion on human adipose tissue. Preliminary studies of the in vivo effect of an intravenous fat emulsion on adipose tissue prostaglandin metabolism have been performed in pediatric patients. A 20% safflower oil emulsion was administered to five subjects ranging in age from 4 to 12 years. These patients required TPN for a variety of reasons. After informed consent was obtained, adipose tissue samples were taken from the subjects before and two and four weeks after treatment. The subjects received the standard TPN regimen with a 20% intravenous fat emulsion for the duration of the study. The doses of lipid ranged from 0.5 to 2 g/kg body weight. Adipose tissue was used to determine fatty acid profiles and in in vitro experiments to measure prostaglandin production in a perifusion apparatus as described above. Samples were extracted overnight at 4 C in chloroform/methanol (2:1, v/v) (Fisher Scientific, Pittsburgh, Pennsylvania). Solvents had been previously redistilled. The resultant samples were methylated using a modification of Glass's method (17). Samples were then analyzed using a Hewlett Packard 5750 Gas Chromatograph equipped with a Hewlett Packard 3390A Reporting Integrator (Avondale, Pennsylvania). A modification of the temperature programming method of Mahadevan and Zieve was used (18).

RESULTS

The results depicted in Figure 1 demonstrate the production of PGE_2, 6-keto $PGF_{1\alpha}$ and TXB_2 by human adipose tissue. There was no significant difference in prostaglandin production between the two columns during the baseline period. The infusion of arachidonic acid bound to albumin caused an immediate, significant increase in the production of PGE_2, which remained elevated throughout the balance of the experiment (800-1950% change). The percent increases in production of both

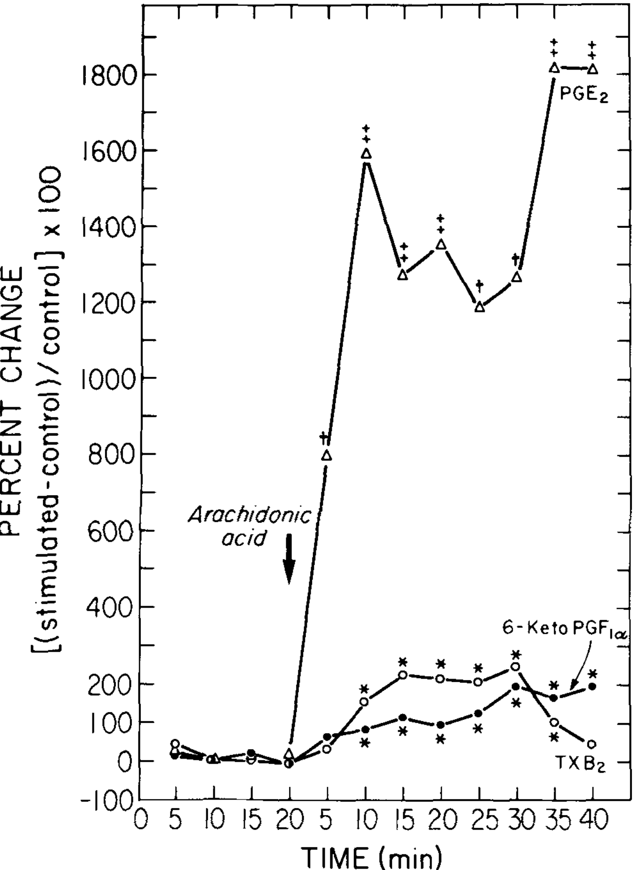

FIG. 1. Effect of arachidonic acid bound to albumin on human adipose tissue prostaglandin production. Adipose tissue was suspended in two identical perifusion apparatuses, and after a 20-min baseline period arachidonic acid bound to albumin was infused. The collected fractions were analyzed for PGE_2, 6-keto $PGF_{1\alpha}$ and TXB_2 by radioimmunoassay. Data was standardized and compared to zero by t-test. Results indicate that human adipose tissue obtained by needle aspiration can rapidly convert arachidonic acid to PGE_2, 6-keto $PGF_{1\alpha}$ and TXB_2. Each data point represents a mean level of the specific prostaglandin assayed. Standard error bars were omitted for clarity. *, $p < .05$; †, $p < .01$; and ††, $p < .001$.

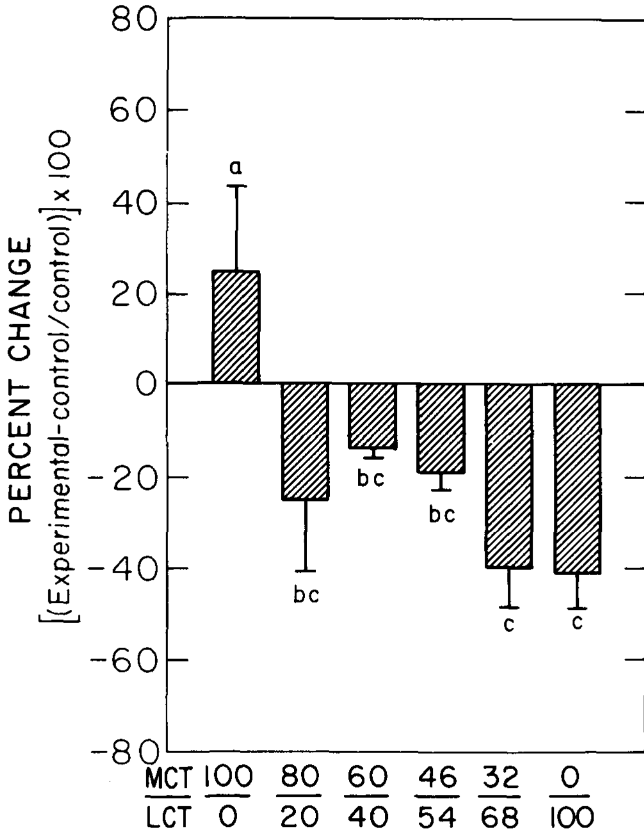

FIG. 2. Effect of physical mixtures of 20% lipid emulsions containing medium (MCT) and/or long chain (LCT) triglyceride on the production of thromboxane (TXB_2) by human adipose tissue. The percentage of MCT and/or LCT varied from 0% to 100%. This altered the concentration of linoleic acid in the incubation media from 0% to 54%. Data was standardized to percent change in activity compared to the control to account for individual variation within each experiment (n = 4). Data are expressed as means ± SEM and were compared by analysis of variance with post-hoc comparisons by Duncan's multiple range test. Means with different letters are statistically different from one another at p < .05.

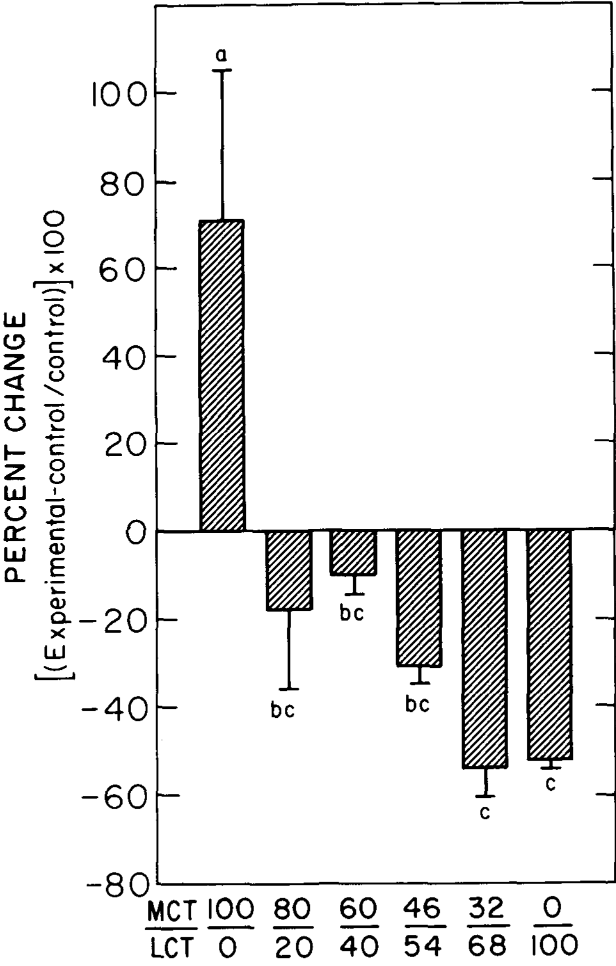

FIG. 3. Effect of physical mixtures of 20% lipid emulsions containing medium (MCT) and/or long chain (LCT) triglyceride on the production of prostacyclin (6-keto $PGF_{1\alpha}$) by human adipose tissue. The percentage of MCT and/or LCT varied from 0% to 100%. This altered the concentration of linoleic acid in the incubation media from 0% to 54%. Data was standardized to percent change in activity compared to the control to account for the individual variation within each experiment (n = 4). Data are expressed as means ± SEM and were compared by analysis of variance with post-hoc comparisons by Duncan's multiple range test. Means with different letters are statistically different from one another at p < .05.

6-keto $PGF_{1\alpha}$ and TXB_2, although not as immediate or dramatic, were also statistically significant (55–209% change). The results demonstrated that human adipose tissue produced the prostaglandins PGE_2, 6-keto $PGF_{1\alpha}$ and TXB_2 in vitro in significant quantities and could alter their production in response to the exogenous EFA arachidonate. This observation led us to investigate other possible uses of this model that would have direct implications on the nutrition of hospitalized patients.

In vitro effects of lipid emulsions on adipose tissue. To control for individual variation in basal production of prostaglandins in each experiment, the data was transformed to reflect the percent change in activity relative to the control flasks, which had no lipid added. The following formula was used: % change = (experimental-control)/control × 100. Comparisons of these data were made by analysis of variance using post-hoc comparisons by Duncan's multiple range test (19) and are summarized in Figures 2–4. The effect of MCT and/or LCT physical mixtures of lipid emulsions on the production of TXB_2 (Fig. 2), 6-keto $PGF_{1\alpha}$ (Fig. 3) and PGE_2 (Fig. 4) by human adipose tissue yielded similar results. Tissue incubated with MCT alone demonstrated a percent increase in the three prostaglandins assayed relative to the control. The addition of LCT, thereby increasing the percentage of linoleic acid, yielded a significant percent decrease in prostaglandin production compared to the control. Therefore, increasing the percentage of LCT (linoleic acid) in the incubation media caused stepwise percent decreases in the production of TXB_2, 6-keto $PGF_{1\alpha}$ and PGE_2.

In vivo effects of a lipid emulsion on human adipose tissue. Subjects 1, 4 and 5 had malnutrition secondary to a more serious disease process. Subjects 2 and 3 had existing inflammatory gastrointestinal disease that had progressed to the point that withholding enteral feedings was indicated. However, subject 2 was relatively well nourished compared to the other individuals studied. The effect of intravenous fat administration on fat cell size is illustrated in Figure 5. All five subjects demonstrated an increase in fat cell size after two and four weeks of

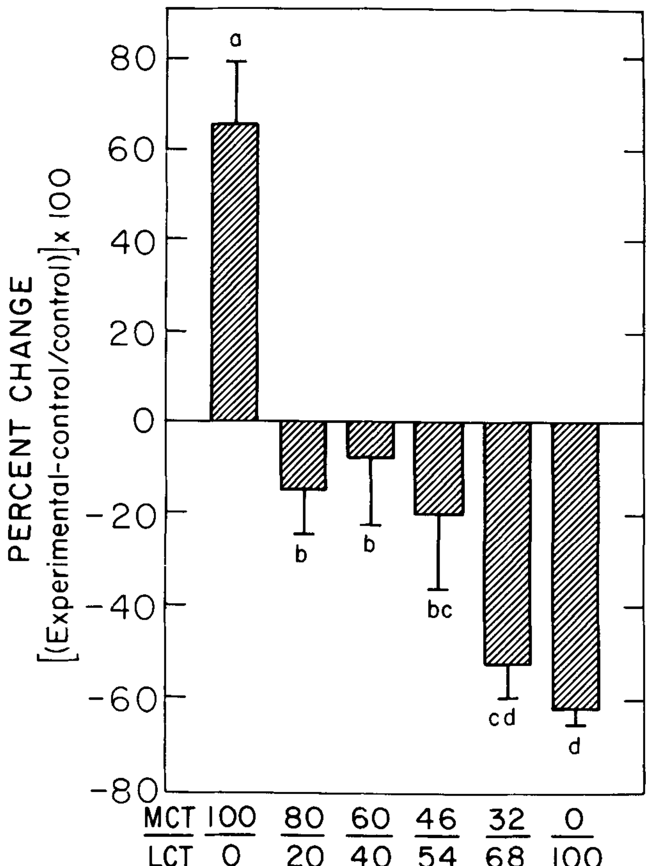

FIG. 4. Effect of physical mixtures of 20% lipid emulsions containing medium (MCT) and/or long chain (LCT) triglyceride on the production of prostaglandin E_2 by human adipose tissue. The percentage of MCT and/or LCT varied from 0% to 100%. This altered the concentration of linoleic acid in the incubation media from 0% to 54%. Data were standardized to percent change in activity compared to the control to account for the individual variation within each experiment (n = 4). Data are expressed as means ± SEM and were compared by analysis of variance with post-hoc comparisons by Duncan's multiple range test. Means with different letters are statistically different from one another at $p < .05$.

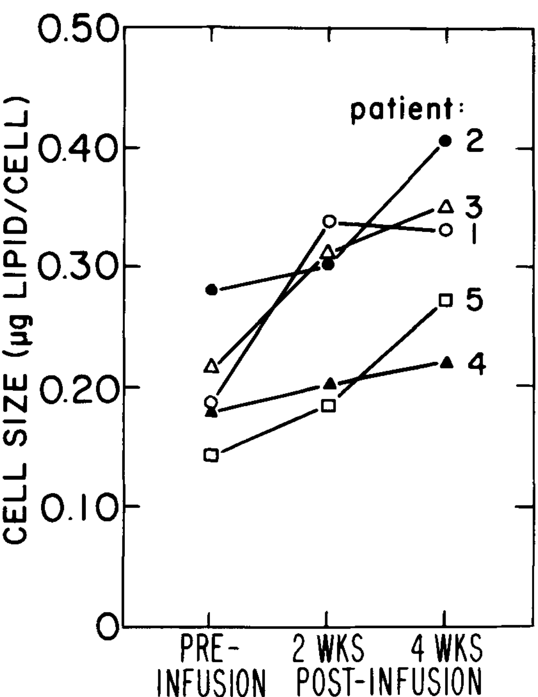

FIG. 5. Effect of intravenous fat administration on fat cell size in five pediatric subjects receiving total parenteral nutrition. Adipose tissue was obtained from the patients by needle aspiration before and after 2 and 4 weeks of treatment. Mean fat cell size (expressed as µg lipid/cell) increased as subjects gained weight over the course of treatment.

treatment. Although this observation must be linked with the fact that patients were gaining weight while receiving TPN, it is of interest that there was a significant increase in their fat cell size. The effect of intravenous fat administration can also be observed on the distribution of fatty acids found in the fat depot. The distribution of fatty acids is expressed as area percent. The upper half of Figure 6 illustrates the changes in linoleic acid levels during treatment. Pretreatment levels in the fat tissue ranged from 8% to 28%. These levels seemed to increase after two and four weeks of treatment. As illustrated in the lower half of Figure 6, the levels of arachidonic acid seemed to increase concomitant with the changes in linoleic acid levels. Therefore, intravenous fat administration with an emulsion high in linoleic acid increased the enrichment of EFA found in the adipose tissue depot in a relatively short time.

The effect of this fat emulsion on adipose tissue prostaglandin production is illustrated in Figure 7. Baseline production of 6-keto $PGF_{1\alpha}$ ranged from 4 to 272 pg/20 min/10^6 fat cell, whereas the levels of TXB_2 ranged from 323 to 4125 pg/20 min/10^6 fat cells. Effects of the infusion of lipid emulsion were variable depending on the subjects. Subjects 1 and 3 demonstrated significant increases in the production of both 6-keto $PGF_{1\alpha}$ and TXB_2 after two and four weeks of treatment. Subjects 2 and 4 demonstrated significant decreases in the production of both 6-keto $PGF_{1\alpha}$ and TXB_2 after two and four weeks of treatment. The production of 6-keto $PGF_{1\alpha}$ by subject 5 increased after two weeks, but significantly decreased after four weeks. The production of TXB_2 by subject 5 significantly decreased after 2 and 4 weeks of treatment. The results demonstrate that the production of the prostaglandins 6-keto $PGF_{1\alpha}$ and TXB_2 by adipose tissue in patients receiving intravenous fat emulsions can be variable and may be affected by the patients' disease and subsequent clinical course.

DISCUSSION

Adipose tissue once was considered an inert tissue reserve, where lipid droplets could cluster between connective tissue. Functionally, it was considered to be solely a means of body protection and thermal insulation. The pioneering studies of Wertheimer and Shapiro (20) demonstrated that the tissue had high levels of metabolic activity and played a central role in carbohydrate metabolism. Furthermore, they demonstrated that the tissue responded to a wide variety of humoral, hormonal, neural and nutritional factors (21).

The function of adipose tissue is the storage of metabolic fuel as triglyceride during times of caloric plenty,

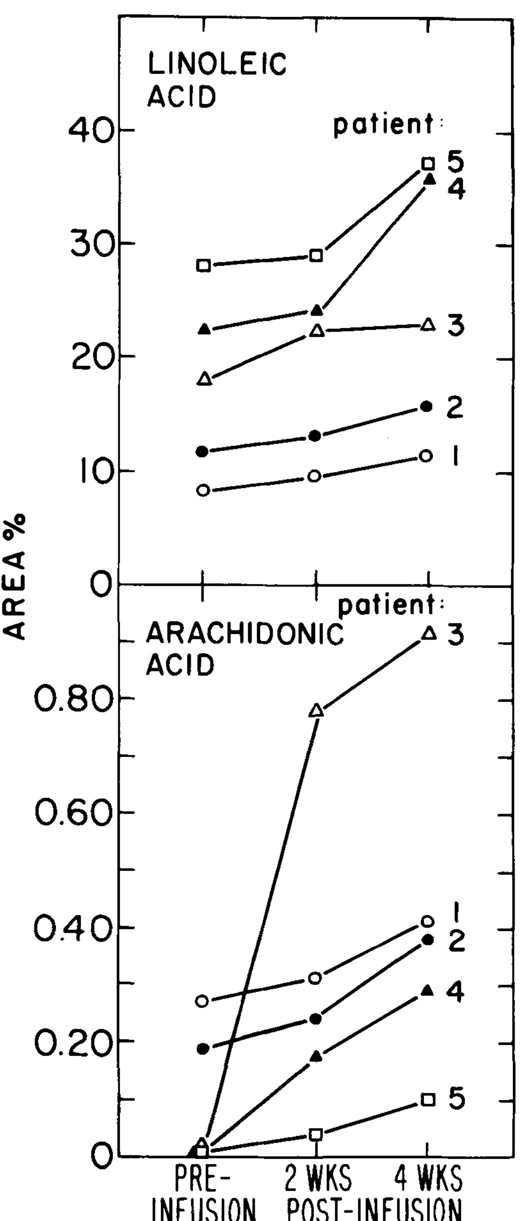

FIG. 6. Effect of infusion of an intravenous fat emulsion high in linoleic acid (77%) on the essential fatty acid profile found in adipose tissue. Five pediatric subjects received a 20% safflower oil lipid emulsion as part of a total parenteral nutrition regimen. Levels of linoleic and arachidonic acids found in adipose tissue are expressed as an area percent of all fatty acids analyzed. Adipose tissue samples were obtained before and after 2 and 4 weeks of treatment. Results demonstrate that intravenous fat infusions significantly increased the percentages of linoleic and arachidonic acids found in adipose tissue.

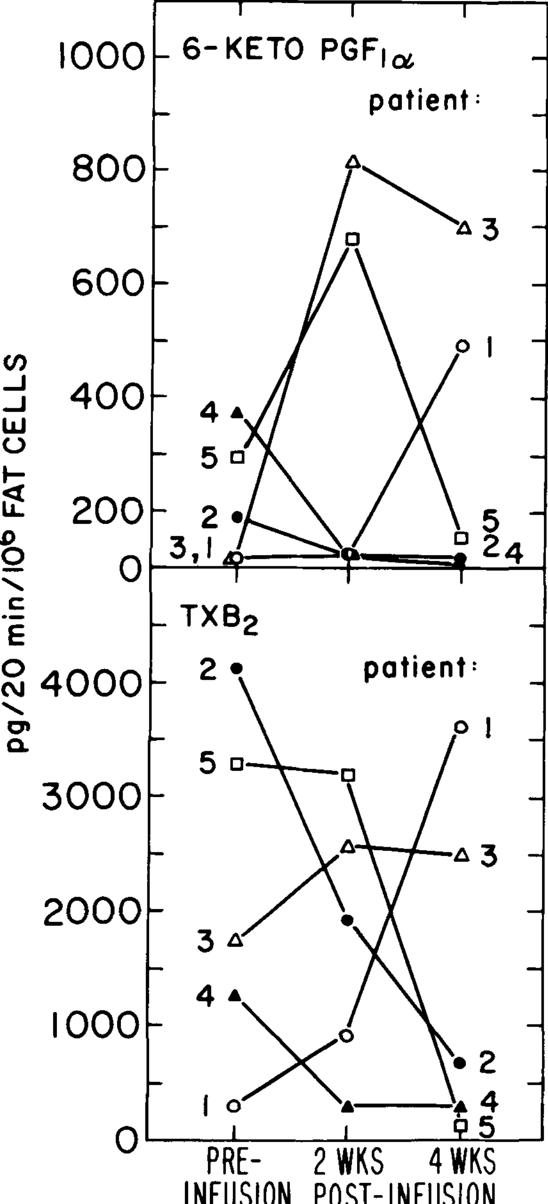

FIG. 7. Effect of infusion of an intravenous fat emulsion high in linoleic acid (77%) on adipose tissue prostaglandin production in five pediatric subjects who received a 20% safflower oil lipid emulsion as part of a total parenteral regimen for 4 weeks. Adipose tissue samples were obtained by needle aspiration before and after 2 and 4 weeks of treatment. The samples were suspended in a perifusion apparatus, and the resulting perfusate was analyzed for the prostaglandins prostacyclin I_2 (measured as 6-keto $PGF_{1\alpha}$) and thromboxane A_2 (measured as TXB_2). Results demonstrate that intravenous fat administration has an unpredictable effect on production of prostaglandins by adipose tissue. This may reflect the condition and clinical course of the patients studied.

and the release of that energy when required. In humans, triglycerides represent the major caloric reservoir of the body compared to the other potential body fuel components, i.e., glycogen and lean body mass. Cahill states in his classic review on starvation (22) in man, "Lipid synthesis and storage are prerequisites to survival in species in which mobility is critical." Therefore, the successful organism must be able to tightly regulate the uptake and release of lipid depending on exogenous and endogenous caloric requirements. The storage and mobilization of fat by adipose tissue is a process that is carefully regulated by several metabolic processes, including de novo fatty acid synthesis of lipids, esterification of long chain fatty acids to activated glycerol and hormone-stimulated lipolysis. Adipose tissue can synthesize long chain fatty acids from glucose and can esterify these fatty acids, as well as those derived from the diet in chylomicrons, to α-glycerophosphate to form triglyceride. This activity is

stimulated by availability of substrate and to a certain degree is regulated by circulating insulin levels. Insulin plays an anabolic role in relation to adipose tissue and can stimulate glucose oxidation, de novo fatty acid synthesis and triglyceride biosynthesis and can inhibit fatty acid breakdown (reviewed in ref. 23).

Lipolysis in adipose tissue consists of the breakdown of stored triglyceride by specific hormone-sensitive lipases and subsequent release of glycerol and free fatty acids (FFA) into the plasma. There is virtually no recycling of glycerol in adipose tissue because the enzyme glycerolkinase is absent. However, FFA may or may not be recycled (23). Virtually every hormone except insulin has been shown in in vitro models to stimulate lipolysis.

The balance between triglyceride storage and mobilization, and therefore the size of the adipose tissue depot, is a function of the overall supply of calories to the organism relative to its energy requirements. During periods of starvation, adipose tissue releases fat in the form of FFA. This is evident from the fact that plasma FFA levels are high in blood vessels draining areas rich in adipose tissue (24). Furthermore, plasma glycerol concentrations increase proportionally to changes in plasma FFA levels, suggesting complete hydrolysis of adipose tissue triglyceride prior to its mobilization (25–27). During feeding or refeeding in starved organisms, there is a rapid fall in plasma FFA levels due to a decrease in lipolysis. Therefore, during periods in which energy balance is positive, there is an increase in deposition of fatty acids as triglyceride in the depot.

The triacylglycerol stores in adipose tissue are continually undergoing lipolysis and reesterification. The net effect is that there is a continuous turnover of fatty acids in the neutral lipid pool. Therefore, the composition of the stored triglyceride is sensitive to changes in dietary fat intake. The intake of dietary fat leads to modification of the profile of fatty acids found in the adipose tissue depot. This modification begins the first time a newborn is fed (28) and continues throughout the life cycle. A number of investigators have demonstrated that adipose tissue fatty acid composition generally reflects an overall pattern of the long-term intake of dietary fatty acids (28–31). Adults fed diets high in linoleic acid double their proportion of this fatty acid found in their adipose tissue (from 11% to 22%) in at least three years (31). Similarly, if one feeds an odd chain fatty acid to animals, there is an increased enrichment of this fatty acid in adipose tissue over time (32–34). Therefore, one can modify the adipose tissue fatty acid profile by modifying enteral fat intake. This observation concerning the effect of the enteral fat intake led us to consider the possible effects of parenterally administered lipid.

The investigation of the effects of lipids, delivered in vivo or in vitro, on human adipose tissue, has demonstrated that fatty acid chain length and unsaturation have a direct effect on its metabolism. These effects include alterations in prostaglandin production, cell size and fatty acid profile. A number of investigators have previously demonstrated that dietary fats can mediate prostaglandin synthesis. Diets high in linoleic acid or with an increased polyunsaturated to saturated ratio generally yield an increase in prostaglandin production (35–40). This is in contrast to studies in which animals have been made EFA-deficient. In these experiments prostaglandin synthesis decreases overall because of decreased precursor availability and the competitive inhibition of the enzymes responsible for their synthesis (41,42). In addition, the abnormal fatty acid that accumulates during EFA deficiency, 5,8,11-eicosatrienoate (20:3ω9), cannot be used as a substrate for prostaglandin production (43,44) and has been shown to be an allosteric inhibitor (45).

Studies in which human volunteers received infusions of intravenous lipid preparations (safflower oil) demonstrated a significant increase in the production of urinary PGE_2 and PGI_2 compared to controls receiving saline (46). Therefore, in healthy volunteers, it is apparent that increased levels of linoleic acid generally increase the production of endogenous prostaglandins. The effect of intravenous fat on human adipose tissue prostaglandin production is less clear. Indeed, in our population there were obvious changes in cell size and fatty acid profile of the adipose tissue in the subjects studied. This is evidence that intravenous fat was indeed being taken up by the adipose tissue. This is supported by the findings of Rossner (1), who demonstrated that fat emulsions are principally removed from the bloodstream by adipose tissue and skeletal muscle. The reasons for the variability in adipose tissue prostaglandin production by these subjects, before and after treatment, are less clear and require further elucidation.

The inhibitory action of the high levels of linoleic acid found in the LCT emulsions is not unexpected. Jeremy et al. (47), using aortic rings from rats, tested whether the type and amount of fatty acids affected the conversion of arachidonic acid to prostacyclin I_2 (measured as 6-keto $PGF_{1\alpha}$). Generally, an increase in the fatty acid concentration caused a dose-dependent inhibition of prostacyclin synthesis. Linoleic and linolenic acids were the most inhibitory and palmitic the least. Although when linoleic acid is given orally or parenterally it appears to stimulate prostaglandin synthesis, its presence in incubation experiments at concentrations of 0.5 to 3.0 mmol/l is strikingly inhibitory. It can be postulated that its similar structure to arachidonic acid may cause a competitive inhibition of the cyclooxygenase, thereby causing a net decrease in prostaglandin production. Therefore, in this model system the triglyceride mixtures highest in linoleic acid may exert a pharmacological rather than a physiological effect.

The currently available fat emulsions provide linoleic acid (18:2ω6), an EFA, in concentrations that range from 54–77%. These levels far exceed the recommendation of the Food and Nutrition Board of the National Academy of Sciences that at least 3% of total energy be provided as polyunsaturated fat (48). Furthermore, in the intravenous fat routinely administered, the amount of linoleic acid is above the recognized average intake. This is approximately 6% of total energy intake (48). This observation prompted us to look at the in vitro and in vivo consequence of varying concentrations of linoleic acid on the production of prostaglandins by human adipose tissue.

Therapeutically, lipid emulsions have been viewed as a source of energy or a means of preventing or ameliorating EFA deficiency. However, the potential effect that this substrate, high in linoleic acid, has on endogenous prostaglandin metabolism has been ignored. Adipose tissue, which plays a key role in the regulation of whole

body lipid metabolism, may represent a useful model in elucidating this information. It is evident from the studies described that the type of fat calorie delivered, i.e., the chain length and degree of saturation, may have a distinct pharmacological activity elicited through the prostaglandin pathway. Theoretically, one could design lipid emulsions with a desired pharmacological activity to be given to patients with specific disease entities. Although it is arguable that a fat calorie is always a fat calorie, it is apparent that these substrates can cause changes in bioactivity when adipose tissue is used as a target organ. There is great potential for developing a new generation of lipid emulsions with desired pharmacological activity to treat hospitalized patients, and human adipose tissue may represent a useful model in determining their bioactivity.

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Role of Brown Adipose Tissue in Thermogenesis Induced by Overfeeding a Diet Containing Medium Chain Triglyceride[1]

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The role of brown adipose tissue in the mechanism of medium chain triglyceride (MCT)–induced thermogenesis was investigated. Under anesthesia, the interscapular brown adipose tissue (IBAT) was excised in male Sprague-Dawley rats, and the animals were fitted with gastrostomy tubes. After a 10-day recovery period, the animals were divided into two groups: one group received a diet containing MCT as 50% of calories, and the other group received an isocaloric diet containing long chain triglyceride (LCT). The diets were fed for 6 wk at a level of calorie intake that was 150% of the ad libitum intake of a parallel control group. During the last week of the study, resting and norepinephrine (NE)-stimulated O_2 consumption and CO_2 production were measured in a Noyons diaferometer. At the end of 6 wk, the animals were weighed and killed. The individual fat pads were dissected and weighed, and an aliquot of the right retroperitoneal fat pad was used to measure adipocyte size and number. The results showed that body weight and adipocyte size (but not adipocyte number) were significantly smaller in the MCT-fed compared to the LCT-fed animals. Resting as well as maximal NE-stimulated oxygen consumption values were significantly higher in the MCT-fed than the LCT-fed rats. It is concluded that the enhanced thermogenesis induced by MCT persists despite the absence of IBAT and that the phenomenon is likely related to more extensive oxidation of MCT- in contrast to LCT-derived fatty acids, thus leading to increased oxygen consumption, enhanced dissipation of energy as heat and diminished efficiency of weight gain and deposition of body fat.
Lipids 22, 442–444 (1987).

Brown adipose tissue (BAT) is currently implicated in the regulation of energy balance. In cold-adapted rats, hypertrophy and hyperplasia of BAT occur. Basal and norepinephrine (NE)-stimulated oxygen uptakes of cold-adapted rats increase to a great extent above the values for warm-acclimated control (1,2). This dramatic increase in nonshivering thermogenesis has been attributed to the activity of BAT and its unique capacity to uncouple oxidative phosphorylation leading to production of energy as heat rather than as ATP (3). The evidence for BAT as the site for nonshivering thermogenesis is very strong in rodents, although exceptions have been reported from studies on Djungarrian hamsters, where extensive excision of BAT had no significant effect on the respiratory response of the animal to infusion with NE (4). Recently, close similarities were reported to exist between the thermogenic response of cold-adapted and "cafeteria-fed" rats (5). Voluntary overfeeding of a highly palatable "cafeteria" diet was shown to be associated with an increase in the wet weight of interscapular BAT, enhanced oxygen consumption capacity and improved response to NE, leading to a lower efficiency of weight gain as compared to normal ad libitum feeding. The increase in oxygen consumption has been attributed to the increase in BAT content and activity (2,6,7). The role of BAT in this observed diet-induced thermogenesis is still a matter of controversy (8,9). Previous studies carried out in our laboratory have demonstrated that overfeeding adult rats 50% above maintenance with a diet containing medium chain triglyceride (MCT) leads to a higher basal as well as NE-stimulated oxygen consumption as compared to overfeeding an isocaloric diet containing long chain triglyceride (LCT). This increase in metabolic rate in MCT-fed rats was accompanied by less deposition of body fat in the MCT-fed than in the LCT-fed rats (10). The present study explores the possibility that interscapular brown adipose tissue (IBAT) may be involved as a mediator in curtailing fat deposition and in the thermogenic effect leading to diminished weight gain in rats in response to MCT feeding.

METHODS AND MATERIALS

Ten male Sprague Dawley rats were anesthesized with chloral hydrate. IBAT was excised, and the animals were then fitted with gastrostomy tubes. The rats were left to recover for a period of 10 days with ad libitum feeding and free access to water. After recovery, the rats were divided into two groups and fed via gastrostomy tube: one group received the MCT diet providing 4 kcal/ml, and the other group an isocaloric diet containing LCT. The composition of the two diets is shown in Table 1. The diets were fed twice daily for 6 wk at a level of calorie intake that was 150% of the intake of a parallel control group of six additional animals fed ad libitum. During the last

TABLE 1

Composition of the Diets

	MCT diet		LCT diet	
	% Wt	% Cal	% Wt	% Cal
MCT	25.0	45.0		
LCT (corn oil)	2.5	5.0	26.0	50.0
Casein	15.2	13.5	15.7	13.4
Sucrose	42.0	36.6	43.0	36.6
Cellulose	12.0		12.0	
Salt mixture	3.0		3.0	
Vitamin mixture	0.3		0.3	

MCT, medium chain triglyceride; LCT, long chain triglyceride.

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week of the study, resting and NE (25 μg/100 g)-stimulated O_2 consumption and CO_2 production were measured using a Noyons diaferometer (Model MG4, 724E, Kipp and Zonen, Delft, Holland). The temperature within the metabolic chamber was 22.5 C. The resting metabolic rate (RMR) was measured over a 2-hr period starting 2 hr after the morning feeding. NE was injected subcutaneously immediately after RMR measurements were obtained. The metabolic rate was calculated from the O_2 consumption and CO_2 production data generated from the diaferometer. The caloric equivalent of the oxygen consumed in 24 hr was derived from standard tables, corrected for the respiratory quotient. At the end of 6 wk, the animals were weighed and killed by decapitation. Individual fat pads were dissected out and weighed from epididymal, retroperitoneal, omental and dorsal fat depots according to the method of Johnson and Hirsch (11). Total dissectible fat was computed by adding the weights of the individual fat pads. Fat cell size and number were measured from a sample of the right retroperitoneal fat pad using the photomicrographic method of Lavau et al. (12). The data were analyzed statistically by the independent t-test for two means.

RESULTS

At the end of the 6-wk feeding period, the body weight (mean ± SE) of the MCT-fed group was 422.4 ± 4.7 g and that of the LCT-fed group was 439.5 ± 5.1 g. Body weight gain was significantly lower ($p < .025$) in the MCT-fed rats (152.4 ± 5.1 g), compared to those fed the LCT diet (169.5 ± 4.9 g).

Adiposity. Table 2 shows that all fat depots were significantly smaller in rats fed the MCT diet compared to rats fed the LCT diet. Total dissectible fat measured as the combined weights of all four depots was 25% less ($p < .001$) in the rats fed MCT (30.2 g ± 0.55) than in those fed LCT (37.2 g ± 0.65). Fat cell size values were significantly reduced in the MCT-fed rats, but fat cell number was not altered.

Thermogenesis. Resting oxygen consumption in MCT-fed rats was significantly higher than that of the LCT-fed group (9.5 ± 0.7 vs 7.2 ± 0.7; $p < .05$). Under maximal NE stimulation, oxygen consumption rose significantly above respective resting values in both MCT- (44%) and LCT (52%)-fed rats. However, despite maximal NE stimulation, the MCT-fed rats showed significantly higher oxygen consumption than the LCT-fed group (13.7 ± 0.5 vs 11.1 ± 0.4; $p < .05$), as shown in Table 3.

DISCUSSION

The present study confirms the thermogenic effect of overfeeding a MCT diet in rats, which we previously reported (10). It further demonstrates that the MCT-enhanced thermogenesis persists even after excision of IBAT. The thermogenic effect of MCT agrees with reports in the literature on the thermogenic effect of other dietary items such as carbohydrates (13,14), proteins (15) and fats (16). Dietary fats were reported recently to differ in their thermogenic response. A diet with unsaturated fatty acids contributing 10% of dietary energy exhibited higher thermogenic response compared to a diet containing equal amounts of saturated fatty acids (17). Excess polyunsaturated fatty acid feeding (corn oil) exhibited lower efficiency of weight gain as compared to beef tallow (18). The above-mentioned studies and our present data suggest that increase in thermogenesis can be effectuated

TABLE 2

Mean Fat Cell Size and Mean Weight (± SE) of Four Main Fat Depots Dissected from Rats with Interscapular Brown Adipose Tissue Excised and Overfed (50% Above Maintenance) for 6 Wk Diets Containing 50% of Calories Either as MCT or LCT

Diet	Fat cell size (μg/cell)	Epididymal (g)	Perirenal (g)	Omental (g)	Dorsal (g)
LCT	0.52 ± 0.010	8.33 ± 0.9	9.33 ± 0.45	4.78 ± 0.15	14.74 ± 1.1
MCT	0.30 ± 0.012	6.51 ± 0.6	7.82 ± 0.24	3.75 ± 0.14	12.1 ± 1.2
p	<0.001	<0.05	<0.05	<0.05	<0.05

MCT, medium chain triglyceride; LCT, long chain triglyceride.

TABLE 3

Resting and Norepinephrine (NE)-Stimulated Oxygen Consumption (Mean ± SE) in Two Groups of Rats with Interscapular Brown Adipose Tissue Excised and Overfed (50% Above Maintenance) for 6 Wk Diets Containing 50% of Calories Either as LCT or MCT

Diet	N	Body wt (g)	Wt gain (g)	Resting O_2 uptake (ml/min/$W^{0.75}$)	Maximal NE-stimulated uptake (ml/min/$W^{0.75}$)
LCT	6	439.5 ± 5.1	169.5 ± 4.9	7.2 ± 0.7	11.1 ± 0.4
MCT	4	422.4 ± 4.7	152.4 ± 5.1	9.5 ± 0.7	13.7 ± 0.5
p		<0.05	<0.025	<0.05	<0.05

MCT, medium chain triglyceride; LCT, long chain triglyceride.

by including a thermogenic dietary component despite equicaloric feeding.

Diet-induced thermogenesis (DIT) reported so far in rats has been attributed to BAT activity or, more specifically, to IBAT activity. The argument that brown fat is activated in overfed animals stems from the observation that DIT has similar manifestations to nonshivering thermogenesis observed in cold-adapted rodents. The weight increases of IBAT after "cafeteria" feeding and after exposure to cold were among the criteria on which such a resemblance was based. The present study shows that enhanced thermogenesis and decreased body fat deposition occur in the absence of IBAT activity. This agrees with many reports in the literature questioning the role of IBAT in DIT (19,20). In comparison with "cafeteria feeding," the increments in oxygen consumption over the resting values were similar in both MCT- and LCT-fed rats. However, the absolute values of NE-stimulated oxygen consumption for MCT were significantly higher than those for LCT. Thus, the higher RMR values of MCT-fed rats compared to LCT-fed rats were maintained after NE stimulation, confirming the effect of the MCT diet on RMR values.

Thus, MCT overfeeding leads to a decrease in body weight gain and body fat compared to LCT overfeeding in rats with IBAT excised. The decrease seems to be due to increased oxygen consumption in MCT-fed rats. IBAT removal did not seem to influence the increased thermogenic response to MCT overfeeding.

NE stimulation of oxygen consumption above basal values did not differ among the MCT- and LCT-overfed rats. This result agrees with the reports by Rothwell and Stock and others (5,6,21) showing that cafeteria feeding in rats is accompanied by more increased responsiveness to NE stimulation than can be entirely accounted for by increased BAT activity. In this study, the IBAT, which constitutes over one-third of the total BAT of the rat (22), was absent, and hence increased responsiveness to NE was not observed. However, absolute values for NE stimulation of oxygen consumption in MCT-overfed rats remained higher than those for LCT-overfed rats, indicating an independent effect of MCT diet on RMR and demonstrating that higher NE-stimulated oxygen consumption values cannot be attributed always to BAT activity. The recent reports in the literature and the results of the present study suggest that, in comparing thermogenic effects of diets, two criteria should be taken into consideration: the caloric content of the diet and the biochemical nature of its components. Both seem to affect the thermogenic response of the animal. Feeding different dietary items while maintaining caloric content seems to affect oxygen consumption, the mechanism of which appears related to the distinct metabolic pathways followed by these dietary components. This thermogenic effect appears to be independent of IBAT activity. However, comparing overfeeding with ad libitum feeding may show an effect on increased responsiveness to NE stimulation, a phenomenon already proven to be accounted for by BAT activity (21). Whether this increased responsiveness to NE stimulation in cafeteria-fed rats is due to either the higher caloric content of the cafeteria diet or to the considerable differences between the components of the cafeteria and the laboratory chow diets needs further investigation.

The question remains: What mechanism is responsible for the increase in oxygen consumption in MCT-fed rats? Following digestion and absorption, MCT-derived free fatty acids bound to albumin are transported directly to the liver via the portal vein (23). In the liver, they are oxidized rapidly, resulting in enhanced ketogenesis as well as CO_2 and ATP production (24). The thermogenic consequences of fatty acid-derived ketogenesis have been demonstrated in isolated rat hepatocytes (25,26). In a recent study in man, the ingestion of MCT, in contrast to LCT-containing meals, was associated with hyperketonemia and increased oxygen consumption (27). The increased synthesis of ketones from acetyl CoA in the liver provides for an additional mechanism for rapid disposal of fatty acids that is not coupled directly to ATP formation, but rather to a "reversed electron transfer" system causing stimulation of hepatic oxygen consumption (26).

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Effects of Fatty Acids on Gap Junctional Communication: Possible Role in Tumor Promotion by Dietary Fat[1]

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Dietary lipids, in particular unsaturated fat, promote the development of many experimental tumors. However, no mechanisms to fully explain these effects have been elucidated. Recent reports, which we summarize here, suggest a role for gap junction-mediated intercellular communication in the process of tumor promotion. We also review tumor-promoting effects of dietary fat on experimental, particularly mammary, carcinogenesis. Our main focus is to review recent data examining the inhibitory effects of unsaturated fatty acids on metabolic cooperation in Chinese hamster V79 cells. These data suggest that inhibition of junctional communication may be involved mechanistically in the promotion of tumors by high levels of dietary unsaturated fat. Finally, potential mechanisms by which unsaturated fatty acids inhibit metabolic cooperation are examined.
Lipids 22, 445-454 (1987).

It has been suggested that environmental factors and conditions may be causally related to many cancers in humans (1,2). In recent years the nutritional aspects of cancer causation have been one of the most intriguing and extensively examined environmental factor(s) in cancer research. Epidemiological evidence has indicated that the incidence of breast cancer among women is related to many nutritional components, in particular the intake of dietary fat. Strong positive correlations between total dietary fat consumption and breast cancer incidence and death rates have been reported (3-11). Other types of cancers also have been related to the intake of dietary fat (3,4,6,8,11).

Such epidemiological evidence has been substantiated by widespread reports that high levels of dietary fat stimulate tumor development in many experimental cancer systems (for reviews, see refs. 6,12,13). The tumor-promoting capacity of high fat diets has been examined extensively. However, any mechanisms to explain these effects remains controversial. Recently, it has been suggested that unsaturated fatty acids may directly influence tumor cell growth processes by inhibiting gap junction-mediated intercellular communication (14). In our report, tumor-promoting effects of dietary fat on experimental cancer are reviewed. In addition, the effects of fatty acids on metabolic cooperation are reviewed, and the possible role inhibition of gap junction-mediated intercellular communication in high fat diet-induced tumor promotion is addressed.

EXPERIMENTAL MAMMARY TUMOR PROMOTION BY DIETARY FAT

The tumor-promoting effects of dietary fat have been well documented using many experimental mammary tumor systems. In 1942, Tannenbaum first described a relationship between dietary fat and the development of spontaneous mammary tumors in mice (15). In the 1960s and 1970s, many laboratories confirmed and extended his initial observation using many different murine mammary tumor models. Elevated levels of dietary fat consistently and significantly stimulate the development of spontaneous, carcinogen-induced and transplantable mammary cancers (Table 1; for reviews, see refs. 6,12,13). In general, animals fed high levels of dietary fat (20% by weight) develop more tumors (increased tumor multiplicity) at a higher frequency (increased tumor incidence) and more rapidly (reduced latency period for tumor appearance) than animals fed a moderate (5%) or low (0.5%) fat diet. The tumorigenesis of virtually every mammary tumor model examined is enhanced by consumption of elevated dietary fat.

TABLE 1

Experimental Mammary Tumor Models That Are Stimulated by Elevated Levels of Dietary Fat[a]

Spontaneous mammary tumors
Mice
Mammary carcinomas in DBA mice
Mammary carcinomas in C3HJ mice
Mammary carcinomas in C3H/St mice
Mammary carcinomas in C3H/Crgl mice
Mammary carcinomas in TM mice
Mammary carcinomas in obese (gold auro thio-glucose-treated) C3H/Crgl mice
Rats
Mammary fibroadenomas in Sprague-Dawley rats
Mammary carcinomas in Sprague-Dawley rats
Carcinogen-induced mammary tumors
Mice
DMBA-induced mammary carcinomas in C3H-A^{vy}fB mice
Rats
DES-induced mammary carcinomas in AxC rats
DMBA-induced mammary fibroadenomas in Sprague-Dawley rats
DMBA-induced mammary carcinomas in Sprague-Dawley rats
NMU-induced mammary carcinomas in Sprague-Dawley rats
NMU-induced mammary carcinomas in Fischer rats
NMU-induced mammary carcinomas in Long-Evans rats
AAF-induced mammary carcinomas in AES rats
X-ray-induced mammary carcinomas in Sprague-Dawley rats
Transplantable mammary tumors
Mice
BALB/c mammary carcinomas
C3H/Crgl mammary carcinomas
C3H-A^{vy}fB mammary carcinomas
Rats
DMBA-induced mammary carcinomas in Wistar/Furth rats
R3230AC mammary carcinomas in Fischer rats

[a]Compiled from refs. 6, 12 and 13.

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The promotion of cancer by high levels of dietary fat is not restricted to, nor specific for, mammary cancer. Development of other experimental cancers is also enhanced by high fat diets. High dietary fat enhances rat colon carcinogenesis induced by 7,12-dimethylbenz(*a*)anthracene (DMBA), dimethyl hydrazine (DMH) and other carcinogens (16–19). Diets with high levels of polyunsaturated fat stimulate rat pancreatic carcinogenesis induced by azaserine (20) as well as the growth of AK3T3 sarcoma cell line tumors in mice (21). Mouse tumors of the integument (22), lymphatic system (23), lung (23) and central nervous system (24) are also promoted by dietary fat.

Most reports suggest that dietary fat enhances mammary tumor development by acting at the promotional stage of the tumorigenic process. The level of fat intake after carcinogen administration ("initiation") is more important in enhancing mammary tumorigenesis than the level before (7,25). Dietary fat exhibits many characteristics of classic tumor promoters as defined by Berenblum (26), including dose-response relationships and reversibility (14,27–29). Also, when high fat dietary treatment is delayed up to 20 wk after carcinogen (DMBA) administration, increased mammary tumor development is still observed in rats fed a high fat diet (30). Treatment of rats with a high dietary fat regimen for equal (3-wk) time periods similarly promotes mammary tumor development, whether the dietary treatment is initiated 0, 2 or 4 wk after DMBA administration (14). Furthermore, Ip and Sinha (31), using explanted mammary glands treated with DMBA, demonstrated that the fat level in recipient rats' diet was more important in subsequent mammary tumor development than that in donor rats' diet. The growth enhancement of many transplantable murine mammary tumors (i.e., already transformed) by high levels of dietary fat also suggests that dietary fat acts at the growth promotional phase of tumorigenesis.

The type of dietary fat is important for the enhancement of mammary tumorigenesis by high fat diets. In general, diets high in polyunsaturated fat appear to promote experimental murine mammary tumor development more than diets high in saturated fat (32–35). Because linoleic acid was the major component of most polyunsaturated fats and oils in these studies, it has been suggested that the role of linoleate itself may be important. Spontaneous mammary tumorigenesis in C3H/St mice is enhanced by increasing the level of linoleic acid at the expense of other fatty acids in the diet (36). Also, linoleic and linolenic, but not oleic, acids appear to be important in the stimulation of DMBA-induced rat mammary tumorigenesis (37). However, Carroll reported that DMBA-induced rat mammary tumorigenesis was similar for rats fed 20% high fat diets containing primarily either oleic acid (olive oil) or linoleic acid (sunflowerseed oil) (7). Also, Dayton et al. (38) found no marked differences in the ability of diets containing high-oleic and high-linoleic safflower oil to promote DMBA-induced rat mammary tumorigenesis. The importance of unsaturation in the promotion of mammary tumorigenesis by dietary fat may be limited, since it appears that once a minimum requirement for unsaturated (essential) fatty acids is met, the total amount of fat, whether saturated or unsaturated, is what determines the influence of high fat levels (7). However, in vitro studies by Kidwell and colleagues have shown that unsaturated fatty acids enhance, whereas saturated fatty acids inhibit, the growth of normal and neoplastic rat mammary epithelium in cell culture (39–41). Also, addition of oleic, linoleic and arachidonic acids stimulates the growth of X563.5 mouse melanoma cells in vitro (42). Preliminary results from our laboratory also indicate that linoleic acid stimulates the growth of V79 Chinese hamster cells in vitro (unpublished data).

The orientation of the double bond as well as the chain length have recently been shown to have an influence on in vivo mammary tumor promotion by dietary fat. Diets with *trans*-unsaturated and with saturated fatty acids are similar in their mammary tumor-promoting capacities (43). Also, a report by Cohen et al. (44) suggests that the mammary tumor-promoting effect of high dietary fat is diminished by substitution of medium chain triglycerides for the more common long chain unsaturated triglycerides.

While the promotion of mammary tumorigenesis by high levels of dietary fat is well documented, any mechanism by which dietary fat promotes mammary tumorigenesis is unclear. Some investigators have suggested that indirect endocrine-related mechanisms (i.e., increased secretion of prolactin and/or estrogen) are involved (45–49). This concept has been reviewed and evaluated (12,13). Reports that consumption of a high fat diet alters circulating levels of prolactin and/or estrogen during certain stages of the estrous cycle have been inconsistent: both increased (47–51) and unchanged (52–55) levels have been reported. In many of these studies, hormone levels were assessed using single-point determinations during different stages of the estrous cycle as well as potentially stressful blood sampling techniques that may have influenced circulating prolactin levels. Also, the small sample size in many reports makes interpretation of observed differences difficult. Aylsworth et al. (55), using in-dwelling right atrial cannulas and repeated blood sampling techniques from "unstressed" animals during the entire estrous cycle, found no difference in circulating prolactin levels in rats fed a high (20%) or moderate (4.5%) fat diet. Indirect endocrine-related mechanisms cannot account for the promotion of mammary tumorigenesis by dietary fat for additional reasons: The development and growth of hormone (pituitary and ovarian)-unresponsive or independent mammary tumors is enhanced by high dietary fat (56–59). Also, the development and growth of many experimental tumors that do not appear to be influenced by the endocrine system are enhanced by high dietary fat (17–21), suggesting that similar mechanisms are involved that do not include the endocrine system. In addition, when circulating levels of estrogen and prolactin are controlled by endocrine and drug manipulations, high fat diets still promote mammary tumorigenesis (60). Also, when serum prolactin levels are similarly elevated in hypothalamic median eminence lesioned rats, mammary tumor development is still enhanced in animals fed a high fat diet (51).

Another proposed mechanism is an increased mammotrophic hormone responsiveness in incipient mammary gland tumor tissue. Cave and Erickson-Lucas (61) have reported that prolactin receptors in NMU-induced mammary tumors are increased in rats fed a 20.0% (high) fat diet compared to a 0.5% (low) fat diet. However, these differences could reflect reduced hormone receptor levels in rats fed the low fat diet, which may be marginally deficient in essential fatty acids. In fact, no differences are

observed in prolactin receptor levels of DMBA- or NMU-induced mammary tumors in rats fed a 20.0% high fat diet or a 4.5% control fat diet (62,63). Ip and Ip (64) reported that estrogen receptor levels in mammary tumors were the same in rats fed moderate (5.0%) and high (20.0%) levels of dietary fat but were reduced in rats fed a low (0.5%) fat diet. Also, progesterone receptor levels were unaffected by dietary fat. Thus, while dietary fat consumption may subtly influence endocrine secretion and/or the responsiveness of incipient mammary tumor tissue to mammotrophic hormones, the processes do not appear to be influenced sufficiently to affect the genesis and/or growth of murine mammary tumors (12,13).

The immune system plays an integral role in the tumorigenic process (65) and has been implicated in mediating the effects of high dietary fat in mammary tumorigenesis. Diets high in polyunsaturated fats suppress immune function (66). Diets high in polyunsaturated fatty acids have been reported to prolong skin allografts in rodents (67), to enhance immunosuppressive therapy following renal transplantation (68), to decrease macrophage activity (69) and to reduce peripheral lymphocyte concentrations (70). However, other reports show no effects on immune activity (71–74). Thus, while mediation of the promotion of mammary tumorigenesis by high levels of dietary fat through immunosuppression is an attractive concept, the complexities of this system and the inconsistencies of the data prevent an adequate assessment of the hypothesis.

Prostaglandins also have been implicated in the promotion of mammary tumorigenesis by dietary fat. Since linoleic and linolenic acids are precursors in the biosynthesis of certain prostaglandins, diets with elevated levels of these fatty acids may provide the substrate for increased synthesis of those prostaglandins. Dupont et al. (75) reported a positive relationship between dietary levels of linoleate and prostaglandin biosynthesis. Also, indomethacin, an inhibitor of prostaglandin biosynthesis (76), can block the stimulatory effects of high dietary fat on mammary tumorigenesis (77,78).

Because certain unsaturated fatty acids stimulate, whereas saturated fatty acids inhibit, the growth of cell cultures of normal and neoplastic rat mammary glands (39–41), dietary lipids may directly stimulate mammary tumor development and growth. The lipid composition of mammary tumors reflects qualitatively and quantitatively the fatty acid content of the diet (78–80). Changes in fatty acid composition of cell membranes may influence cellular biophysical phenomenon, including membrane fluidity, macromolecular mobility, receptor availability, enzyme activation, cyclic nucleotide and prostaglandin biosynthesis and amino acid and carbohydrate transport processes. These changes may provide conditions favorable to inducing cell division or may interfere with the processes that control cell division. Thus, the mitogenic signal that ultimately results in increased mammary tumor development and growth may be the alteration of the lipid composition of the cellular membrane itself.

INTERCELLULAR COMMUNICATION AND TUMOR PROMOTION

The development of many experimental tumors following application of a carcinogen (initiator) at subthreshold levels depends upon subsequent treatment with a "noncarcinogenic" tumor promoter. Classically, when DMBA or another carcinogen is applied to mouse skin at subthreshold doses, a high incidence of local tumors is observed only in animals subsequently treated with a tumor promoter that is noncarcinogenic when applied alone (81,82). This observation has been expanded and adapted to include tumor promotion in other tissues. For example, in mammary carcinogenesis, development of tumors following DMBA or NMU treatment is dependent upon subsequent exposure to mammotrophic hormones (83,84). Removal of pituitary or ovarian influences results in nearly complete suppression of mammary tumor development (85,86). An understanding of how the growth of these initiated, potentially tumorigenic cells is suppressed or, conversely, how tumor promoters are able to reverse this suppression and allow for development of the tumor, would contribute to understanding the tumorigenic process.

One theory to explain suppression of the proliferation of initiated, latent tumor cells is that normal cells surrounding the transformed or initiated foci exert a growth inhibitory influence. Indeed, growth of transformed C3H10T1/2 cells is inhibited when they are cocultured with nontumorigenic "normal" cells in vitro (87,88). This effect is overcome when tumor promoter 12-0-tetradecanoyl-phorbol-13-acetate (TPA) is added to the culture medium (89,90).

Intercellular communication is thought to be involved in a wide variety of developmental processes and in control of cellular growth and differentiation (91–93). Recently it was suggested that intercellular communication may also have a role in tumor promotion (94). A specific type of intercellular communication, metabolic cooperation, involves the passage of low molecular weight and possibly growth-regulating molecules through membrane structures called gap junctions. In 1979, Yotti et al. (95) and Murray and Fitzgerald (96) independently reported that metabolic cooperation between cells in culture is blocked by the classical tumor promoter TPA. Since these initial reports, many other tumor-promoting compounds have been shown to inhibit metabolic cooperation (94,97–107). Table 2 lists compounds that inhibit gap junction-mediated intercellular communication. A diverse range is listed, including naturally occurring compounds, environmental toxicants and/or pollutants, drugs, food additives and nutritionally related compounds. Thus, inhibition of intercellular communication by tumor promoters is not limited to related compounds, nor is it specific to a certain class of compounds. Also, correlations linking the efficacy of these compounds as tumor promoters to their ability to block metabolic cooperation in vitro have been described (94,107). Accordingly, a hypothesis has evolved whereby the enhanced proliferation of "initiated" cells and subsequent tumor development induced by tumor promoters (e.g., TPA) is caused by inhibiting intercellular communication, resulting in a blockade of the transfer of growth inhibitory signals via gap junctions. Increased proliferation of initiated cells in the presence of tumor promoters allows these cells to gain a selective growth advantage and increases the probability for further mutational events, resulting in autonomous, promoter-independent growth (i.e., tumor progression).

TABLE 2

Tumor Promoters That Inhibit Intercellular Communication[a]

	Tumor-promoted tissue
Naturally occurring compounds	
TPA (and other tumor-promoting phorbol esters)	Skin
Teleocidin	Skin
Bile acids (deoxycholic, lithocholic acids)	Colon
T-2 toxin	Esophagus
Aplasiatoxin	Skin
Environmental toxicants and/or pollutants	
2,4,5,2′,4′,5′ Hexabromobiphenyl	Liver
2,4,5,2′,4′,5′ Hexachlorobiphenyl	Liver
Benzoyl peroxide	Skin
Anthralin	Skin
Di-(2-ethyrexyl)-phthalate	Liver
Chlordane	Liver
Cigarette smoke condensates	Skin
Lindane	Liver
2,4-Dinitrofluorobenzene	Skin
DDT-(1.1.1-trichloro-2,2-bis p-chlorophenyl ethane)	Liver
Kepone	Liver
Aldrin	Liver
Dieldrin	Liver
Mirex	Liver
NTA-(trisodium nitrilotriacetate monohydrate)	Kidney
Drugs, food additives	
Phenobarbitol	Liver
Chlorpromazine	Liver
BHT (butylated hydroxytoluene)	Liver
Nutritionally related compounds	
Saccharin	Urinary bladder
Cyclamates	Urinary bladder
Unsaturated fatty acids	Mammary gland, others
Retinoic acid (high concentrations)	Skin

[a]Taken in part from ref. 94.

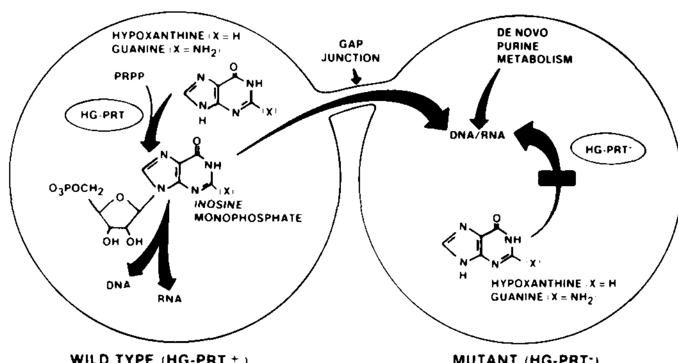

FIG. 1. Diagram illustrating the principle of the V79 Chinese hamster cell metabolic cooperation assay to measure gap junction-mediated intercellular communication (ref. 94).

IN VITRO METABOLIC COOPERATION ASSAY SYSTEM

An in vitro assay system to examine the influence of various environmental compounds on intercellular communication and to study the mechanisms of tumor promotion was developed by Trosko and colleagues (108,109). Gaudin et al. (110) observed that TPA enhanced the recovery of ultraviolet (UV) light-induced 6-thioguanine (hypoxanthine guanine phosphoribosyl-transferase-deficient [HG-PRT$^-$])-resistant Chinese hamster cells in culture. It was subsequently shown by Yotti et al. (95) that the enhancement of recovery of 6-thioguanine (6-TG)-resistant mutant cells was due to a blockade of metabolic cooperation by TPA.

Metabolic cooperation, on which our in vitro assay is based, is the phenomenon in which low molecular weight and possibly growth-regulating molecules are passed from the cytoplasm of one cell to an adjacent cell via membrane structures called gap junctions. The phenomenon is schematically illustrated in Figure 1. HG-PRT is an enzyme involved in the purine salvage pathway. Normal wild-type V79 Chinese hamster cells that contain HG-PRT (HG-PRT$^+$), grown in vitro in culture medium containing 6-TG, take up 6-TG, phosphoribosylate it to a lethal metabolite (6-thioguanosine monophosphate), are unable to proliferate and die. However, V79 Chinese hamster cells that have been mutated by x-irradiation or UV radiation and lack HG-PRT (HG-PRT$^-$) are unable to metabolize 6-TG and therefore continue to proliferate in medium containing 6-TG. (Because HG-PRT is a nonessential enzyme, metabolism of these cells is otherwise normal.) When wild-type (HG-PRT$^+$) V79 cells are cocultivated with mutant (HG-PRT$^-$) V79 cells in medium containing 6-TG, the HG-PRT$^+$ cells take up the 6-TG, phosphoribosylate it and transfer the lethal metabolite (6-TG monophosphate) via gap junctions to the mutant HG-PRT$^-$ cells if they are in physical contact. Transfer of 6-TG monophosphate is dependent upon the presence and proper functioning of gap junctions. Sufficient transfer of 6-TG monophosphate will kill the HG-PRT$^-$ cells. Therefore, recovery of the mutant HG-PRT$^-$ V79 cells is inversely related to the amount of metabolic cooperation between HG-PRT$^+$ 6-TG-sensitive (6-TG^S) and HG-PRT$^-$ 6-TG-resistant (6-TG^R) cells (i.e., increased recovery of HG-PRT$^-$ 6-TG^R cells indicates decreased metabolic cooperation). Addition to the culture medium of chemicals that decrease metabolic cooperation will result in increased recovery of 6-TG^R cells. As stated previously, a number of known tumor promoters inhibit the transfer of 6-TG monophosphate from 6-TG^S to 6-TG^R V79 Chinese hamster cells and increase the recovery of HG-PRT$^-$ cells (i.e., inhibit metabolic cooperation).

Figure 2 summarizes the experimental protocol of the V79 metabolic cooperation assay system. Details of the procedure have been published elsewhere (108,109); they are summarized as follows: Cytotoxicity is initially tested by examining the effect of various fatty acid doses on the colony-forming ability of 100 V79 Chinese hamster cells in 6-cm tissue culture dishes. All fatty acids are dissolved and diluted in 100% ethanol and are added to the cultures about 3 hr after the cells are seeded. Dilutions are made such that the final concentration of ethanol in the culture medium is less than 0.5%. Cultures are incubated in humidified air with 5% CO_2 at 37 C until colonies have grown big enough to be scored visually (usually 7–9 days), with medium changes after three and six days. The culture medium is a modified Eagle's minimal essential

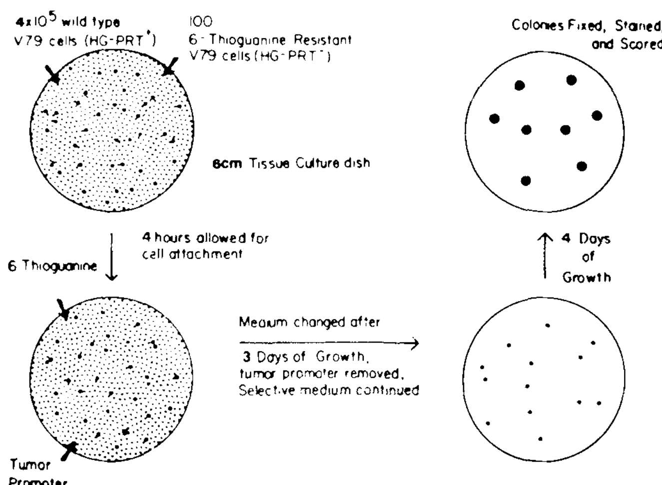

FIG. 2. Diagram describing the experimental protocol of V79 cell metabolic cooperation assay (ref. 94).

medium with Earle's salts, supplemented with 100% increase of all nonessential amino acids, 50% increase in all vitamins and essential amino acids except glutamine, 1.0 mM sodium pyruvate, antibiotics (100 IU penicillin and 100 IU streptomycin per ml medium) and 3% fetal bovine serum. Colonies are rinsed with 0.85% saline, fixed and stained with crystal violet and scored visually.

Once appropriate noncytotoxic dose ranges are established, the effect of various fatty acids on metabolic cooperation is assessed. Wild-type HG-PRT$^+$ 6-TG^S cells are seeded in 6-cm dishes (4×10^5 cells per plate) with 100 HG-PRT$^-$ 6-TG^R cells in 5 ml of culture medium. After 3–4 hr, various doses of the test fatty acids are added. Ethanol is added (final concentration of 0.5%) to a series of plates as a solvent control. TPA (1–2 ng/ml) is added to another series of plates as a positive control. After test chemicals have been added, 6-TG (10 μg/ml) is added to all plates. Cells are cultured for three days, after which the culture medium is changed and replaced with selective culture medium containing only 6-TG (10 μg/ml). Culture medium is changed once again on day 6. Cytotoxicity is confirmed by testing the effect of the same concentrations of fatty acids on the colony-forming ability of 100 6-TG^R metabolic cooperation-deficient (MC$^-$) mutants (111) cocultured with 4×10^5 6-TG^S cells in 6-cm tissue culture dishes in the presence of 6-TG (10 μg/ml). This method allows for more accurate assessment of cytotoxicity since, unlike the previous cytotoxicity evaluation (i.e., 100 6-TG^R cells cultured alone), the cell density conditions are identical to those used in the metabolic cooperation determinations.

FATTY ACID EFFECT ON METABOLIC COOPERATION

In view of the promotion of mammary tumorigenesis by high fat diets and the possible role of inhibition of gap junction-mediated intercellular communication in tumor promotion, a series of experiments was conducted to assess the influence of fatty acids on metabolic cooperation (14,112). It is apparent from the data presented in Figures 3–5 that unsaturated fatty acids inhibit metabolic cooperation at noncytotoxic concentrations, whereas

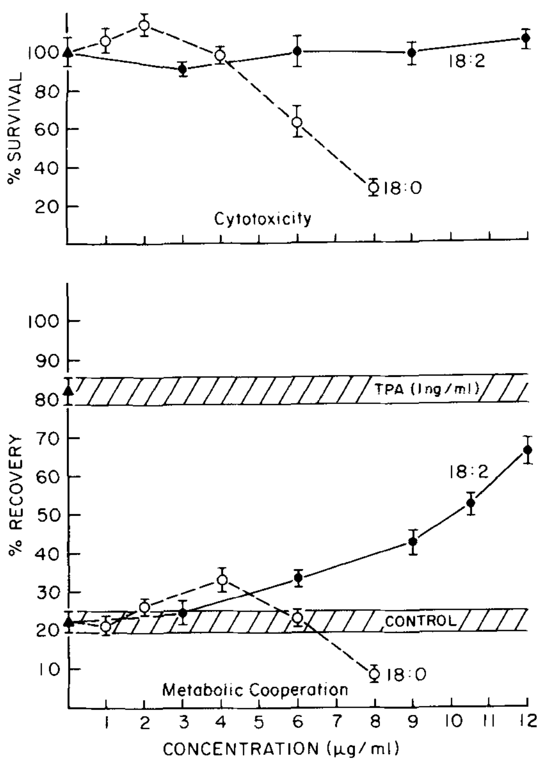

FIG. 3. Effect of linoleic acid (18:2) and stearic acid (18:0) on cytotoxicity (% survival) and on metabolic cooperation (% recovery) between Chinese hamster V79 cells. Negative (solvent) and positive (TPA-treated) controls shown in shaded areas with SEM (Ref. 112).

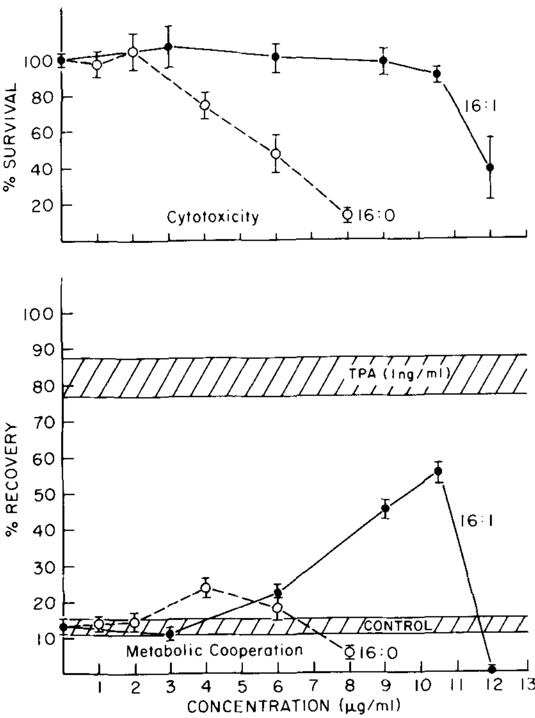

FIG. 4. Effect of palmitoleic acid (16:1) and palmitic acid (16:0) on cytotoxicity (% survival) and metabolic cooperation (% recovery) between Chinese hamster V79 cells (ref. 112).

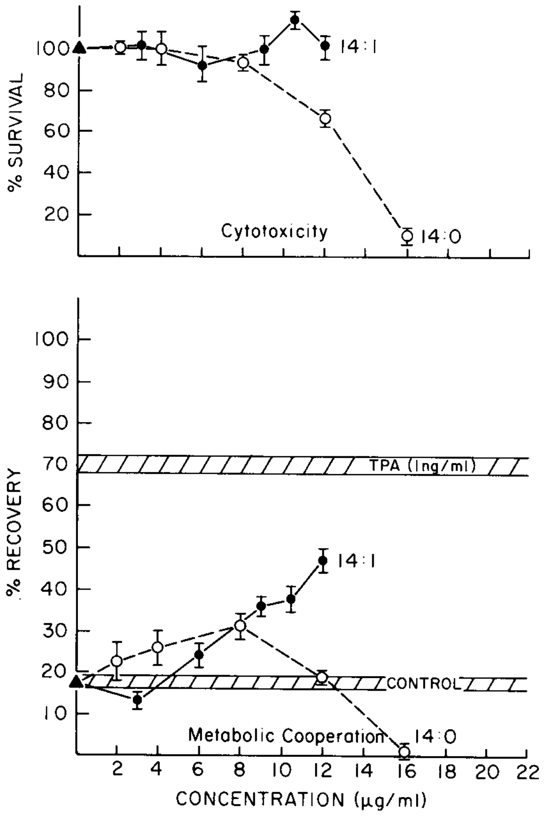

FIG. 5. Effect of myristoleic acid (14:1) and myristic acid (14:0) on cytotoxicity (% survival) and on metabolic cooperation (% recovery) between Chinese hamster V79 cells (ref. 112).

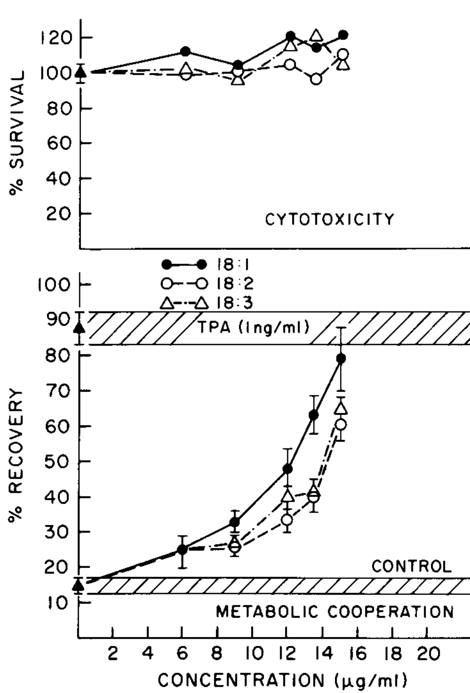

FIG. 6. Importance of the degree of unsaturation on the inhibition of metabolic cooperation by unsaturated fatty acids. Effects of oleic (18:1), linoleic (18:2) and linolenic acids (18:3) on cytotoxicity (% survival) and metabolic cooperation (% recovery) between Chinese hamster V79 cells (ref. 112).

saturated fatty acids fail to do so at any concentration. The unsaturated fatty acids—linoleic (18:2; Fig. 3), palmitoleic (16:1; Fig. 4) and myristoleic (14:1; Fig. 5)—significantly increase the recovery of 6-TG^{R} cells cocultured with 6-TG^{S} cells in a concentration-dependent manner at noncytotoxic concentrations. However, the saturated fatty acids—stearic (18:0; Fig. 3), palmitic (16:0; Fig. 4) and myristic (14:0; Fig. 5)—failed to significantly influence the recovery of 6-TG^{R} cells at noncytotoxic concentrations. Other unsaturated 18-carbon fatty acids, linolenic (18:3) and oleic (18:1), also inhibit metabolic cooperation, resulting in increased recovery of 6-TG^{R} cells (Fig. 6).

To assess the importance of the degree of unsaturation in inhibiting metabolic cooperation by unsaturated fatty acids, the abilities of 18:1, 18:2 and 18:3 to increase 6-TG^{R} cell recovery were evaluated. Figure 6 shows that no relationship exists between degree of unsaturation and inhibition of metabolic cooperation. Oleic acid (18:1) appears slightly more efficacious than linoleic (18:2) or linolenic (18:3) acids, which are about equal, in inhibiting metabolic cooperation.

There is an apparent association between the ability of unsaturated fatty acids to inhibit metabolic cooperation and the carbon chain length. When fatty acids with the same degree of unsaturation (i.e., one double bond) but different chain lengths are compared, those with longer chain lengths inhibit metabolic cooperation more than those with shorter chain lengths. Figure 7 shows that

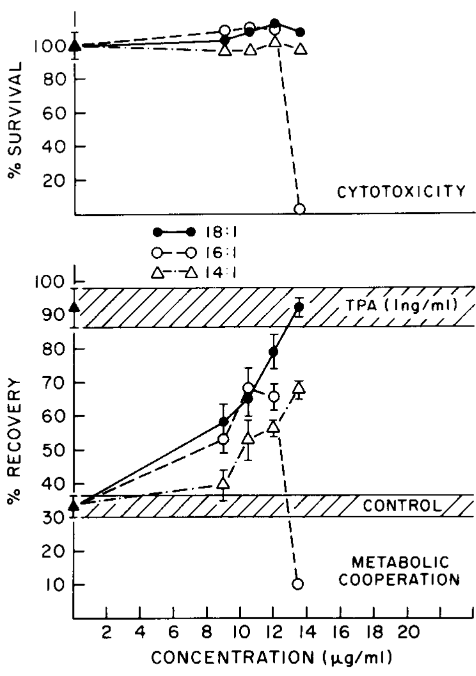

FIG. 7. Influence of carbon chain length on the inhibition of metabolic cooperation by unsaturated fatty acids. Effects of oleic (18:1), palmitoleic (16:1) and myristoleic acids (14:1) on cytotoxicity (% survival) and metabolic cooperation (% recovery) between Chinese hamster V79 cells (ref. 112).

oleic acid (18:1) inhibits metabolic cooperation more than palmitoleic (16:1) or myristoleic (14:1) acids. Palmitoleic acid (16:1) appears to inhibit metabolic cooperation slightly more than myristoleic acid (14:1).

Geometric isomerism also appears important in determining the effect of unsaturated fatty acids on metabolic cooperation. Fatty acids with the *cis*-double bond orientation are more efficacious than the corresponding *trans*-oriented fatty acids. *Cis*-oleic acid (*cis* 18:1) is much more effective than elaidic acid (*trans* 18:1) (Fig. 8), and *cis*-palmitoleic acid (*cis* 16:1) is more effective than palmitelaidic acid (*trans* 16:1) (Fig. 9). However, the differences between *cis* and *trans* 16:1 are much less dramatic than those between *cis* and *trans* 18:1. These data thus suggest that the *cis* double bond orientation appears important in the inhibition of metabolic cooperation by certain unsaturated fatty acids, but less important for other fatty acids.

To further examine the influence of fatty acids on intercellular communication, the effect of medium (11-carbon) and short (6-carbon) chain fatty acids on metabolic cooperation was assessed. Both undecanoic (11:0) and undecylenic (11:1) acids significantly inhibit metabolic cooperation (Fig. 10). However, compared with oleic acid (18:1), the inhibition of metabolic cooperation by 11:0 and 11:1 is apparent only at much higher concentrations. Hexanoic (6:0) and sorbic (6:2) acids do not influence metabolic cooperation at concentrations up to 100 μg/ml (Fig. 11) or 200 μg/ml (data not shown). Thus, it appears that the importance of unsaturation and the ability of fatty acids to inhibit metabolic cooperation is reduced in medium and short chain fatty acids.

CONCLUSIONS

High levels of dietary fat clearly promote many types of experimental cancers and are implicated in the etiology of some human cancers. Many mechanisms, including endocrine-related and immune-mediated ones, have been proposed to explain the promoting influence of high polyunsaturated fat diets on mammary tumorigenesis. A review of available information indicates that a direct influence of dietary fat promoting growth of incipient mammary tumor tissue is the most likely interpretation. One way in which unsaturated fatty acids may directly influence the growth regulation of mammary tumor tissue, and thereby promote tumor development, is by inhibiting gap junction-mediated intercellular communication. This inhibition is associated with many known tumor promoters. Indeed, unsaturated fatty acids such as oleic, linoleic, linolenic, palmitoleic and myristoleic inhibit metabolic cooperation, a process that depends on gap junction-mediated intercellular communication. While there is no apparent relationship between the degree of unsaturation of fatty acids and their ability to inhibit metabolic cooperation, long chain unsaturated fatty acids (i.e., C18) are more effective than medium (C11) or short (C6) chain ones. The *cis* double bond orientation is important for certain fatty acids to inhibit metabolic cooperation. Also, unsaturation is less important in medium and short chain fatty acids than in longer (≥C14) chain ones.

The mechanism by which unsaturated fatty acids inhibit metabolic cooperation is unclear. Incorporation of unsaturated fatty acids into cell membranes may alter

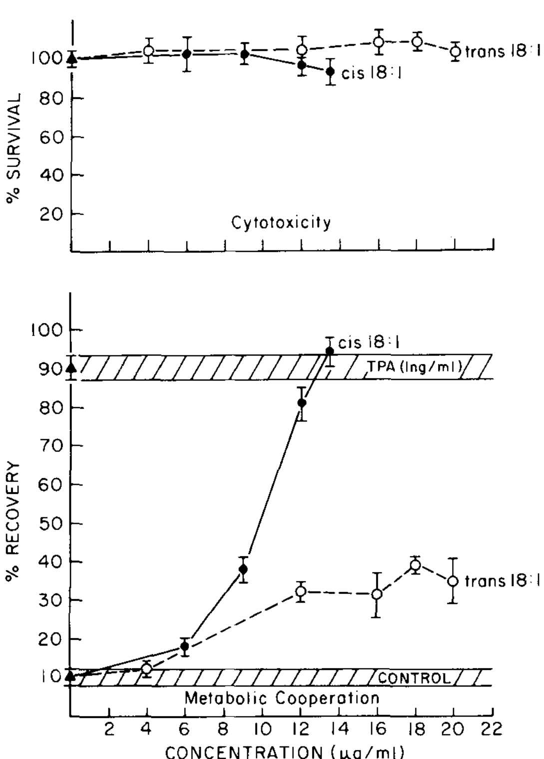

FIG. 8. Importance of *cis*-double bond orientation on the inhibition of metabolic cooperation by unsaturated fatty acids. Effects of *cis*-oleic acid (*cis* 18:1) and elaidic acid (*trans* 18:1) on cytotoxicity (% survival) and metabolic cooperation (% recovery) between Chinese hamster V79 cells (ref. 112).

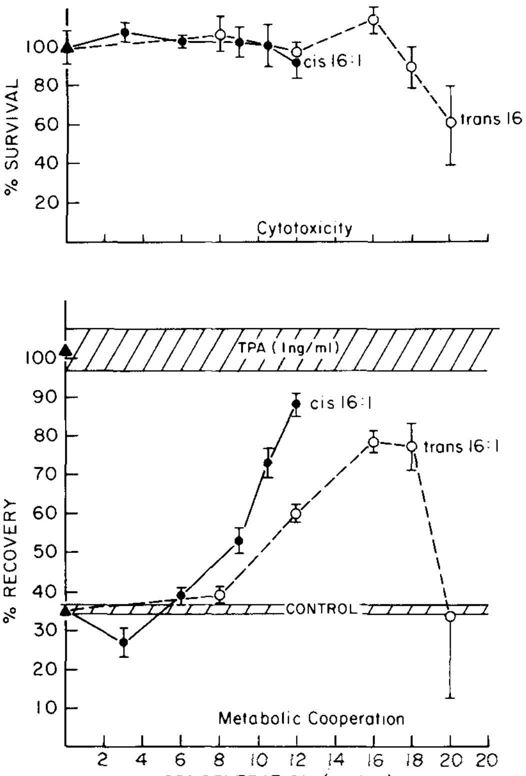

FIG. 9. Importance of *cis*-double bond orientation on the inhibition of metabolic cooperation by unsaturated fatty acids. Effects of *cis*-palmitoleic acid (*cis* 16:1) and palmitelaidic acid (*trans* 16:1) on cytotoxicity (% survival) and metabolic cooperation (% recovery) between Chinese hamster V79 cells (ref. 112).

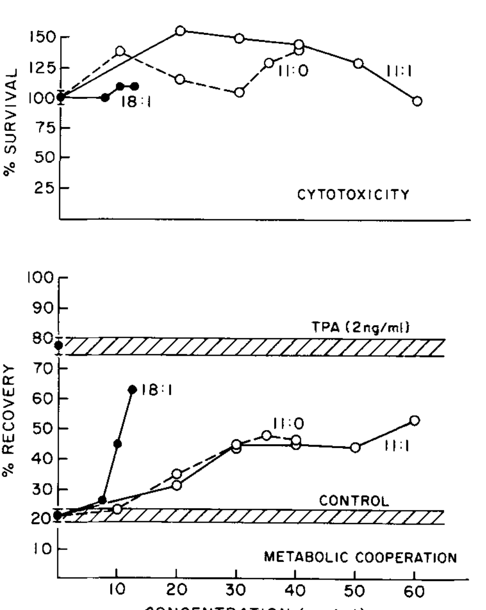

FIG. 10. Effect of undecanoic acid (11.0) and undecylenic acid (11:1) on cytotoxicity (% recovery) and metabolic cooperation (% recovery) between Chinese hamster V79 cells.

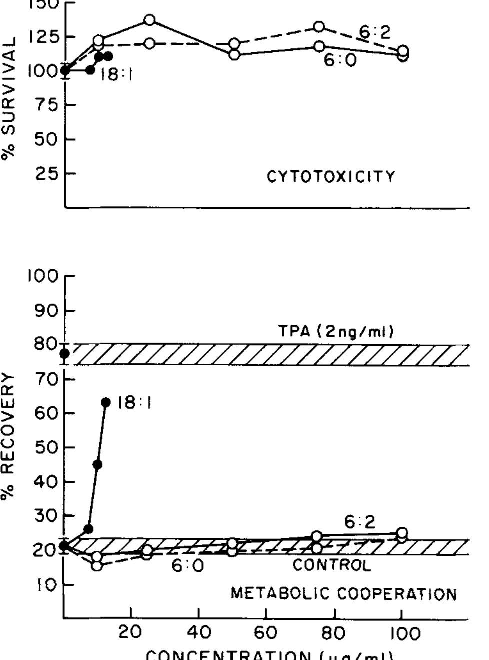

FIG. 11. Effect of hexanoic acid (6:0) and sorbic acid (6:2) on cytotoxicity (% survival) and metabolic cooperation (% recovery) between Chinese hamster V79 cells.

many biophysical properties of the membrane, including membrane fluidity, receptor and macromolecular availability and transport mechanisms. Since gap junctions are membrane structures, changes in the membrane microenvironment could alter their functional capacity. Incorporation of unsaturated fatty acids into the membrane increases fluidity (80), which could result in a destabilization of gap junction structure and thereby cause a decrease in gap junction-mediated intercellular communication (i.e., metabolic cooperation). However, since no greater inhibitory effects were observed with more unsaturated fatty acids (i.e., 18:1, 18:2, 18:3), such mechanisms involving membrane fluidity are unlikely.

Unsaturated lipid may also inhibit intercellular communication by altering enzyme activity, in particular, the Ca^{2+}-activated, phospholipid-dependent, diacylglycerol-sensitive protein kinase C. Protein kinase C is a widely distributed cyclic AMP-independent protein kinase (or group of kinases) recently implicated in mediating many cellular processes, including tumor promotion, in normal and neoplastic tissues (113,114). Its activity is increased by tumor promoters that inhibit intercellular communication (such as TPA) and by diacylglycerol compounds that contain unsaturated fatty acids (113,115-118). Recently, Gainer and Murray (119) and Aylsworth et al. (112) reported that unsaturated diacylglycerol compounds inhibit metabolic cooperation, suggesting a role for protein kinase C in the control of intercellular communication.

Linoleic and linolenic acids may influence the biosynthesis of certain prostaglandins and thereby affect a variety of cellular processes, including intercellular communication (120). However, the inhibition of metabolic cooperation by other unsaturated fatty acids thought to be not involved in prostaglandin biosynthesis (oleic, palmitoleic, and myristoleic acids) suggests that altered prostaglandin biosynthesis is not involved in mediating these effects.

Finally, lipid peroxidation products may also have a role in mediating the inhibitory effects of unsaturated fatty acids on metabolic cooperation. Oxygen free radicals generated by peroxidation of unsaturated fatty acids may cause dysfunction of gap junction structures, thereby reducing gap junction-mediated intercellular communication. However, mechanisms involving lipid peroxidation also are unlikely, because no increased inhibition of metabolic cooperation was observed with fatty acids containing increasing degrees of unsaturation.

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The Influence of Dietary Medium Chain Triglycerides on Rat Mammary Tumor Development[1]

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The N-nitrosomethylurea rat mammary tumor model was used to compare the tumor-promoting effects of a high-fat (HF) diet containing a 3:1 mixture of medium chain triglycerides (MCT) and corn oil with that of a HF and a low-fat (LF) corn oil diet. The serum and tumor lipid content and fatty acid (FA) composition were also determined in the three dietary groups. It was found that the MCT-containing diet failed to promote tumor development compared with the HF corn oil group. Tumor incidence in the HF-MCT group was similar to that of the LF corn oil group (5% fat, w/w), but significantly decreased compared to the HF corn oil group. Total serum cholesterol levels were significantly depressed in the HF corn oil group compared to the HF-MCT and LF corn oil groups. Analysis of serum and tumor FA profiles indicated that the HF corn oil group exhibited approximately twice the amount of linoleic acid (LA) as the other two treatment groups. Differences among the three groups in the major FA metabolite of LA, arachidonic acid, were minimal. These results are consistent with the hypothesis that tumor promotion by dietary fat is more a function of the type than the amount of fat ingested. In addition, they indicate that MCT, due at least in part to their unique structural and physiological properties, exert markedly different effects on mammary tumor development than conventional long chain unsaturated fatty acids.
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Numerous epidemiological and experimental studies suggest that dietary fat is an important determinant of breast cancer risk (1,2). Studies in laboratory animals have shown that the influence of dietary fat is exerted primarily on the promotion stage of mammary carcinogenesis (3), a finding that may be reflected in the fact that the association between fat intake and increased risk is most pronounced in women over 40 (2).

Moreover, experimental studies by us (4), Carroll et al. (3,5-7) and others (8-15) have shown that the kind and amount of fat ingested determine the rate of development of chemically induced and transplantable mammary tumors. In these studies, it was shown that a certain proportion of essential polyunsaturated fatty acids (PUFA) appears to be required for optimal tumor-enhancing effects, and that high-fat (HF) diets containing coconut oil, which consists primarily of short and medium chain saturated fatty acids and a small quantity (<2%) of linoleic acid (LA), lack the ability to promote mammary tumor development compared to HF diets rich in PUFA.

As the above evidence suggests, the degree of saturation and possibly the chain length of constituent fatty acids can modify the effect of HF diets. Hence, it appeared appropriate to test the effects of a specifically designed diet containing high levels of medium chain triglycerides (MCT) on the development of chemically induced mammary tumors. (Medium chain fatty acids [MCFA] are obtained by hydrolysis of coconut oil—6:0 [<2%], 8:0 [7%], 10:0 [6%], 12:0 [50%], 14:0 [20%], 16:0 [8%], 18:0 [2%], 18:1 [5%], 18:2 [0.8%]—followed by fractional distillation of the resulting fatty acid mixture to obtain a mixture of 6:0 [1-2%], 8:0 [65-75%], 10:0 [25-35%], 12:0 [1-2%] fatty acids. The MCFA are then esterified to glycerol in the presence of a Zn catalyst to generate the triglyceride [17].)

In the present study, the N-nitrosomethylurea (NMU)-induced rat mammary tumor model was used to compare the tumor-promoting effects of diets containing low and high levels of corn oil with a HF diet containing a 3:1 mixture of MCT and corn oil. Since dietary MCT have been reported to alter circulating serum lipid levels (16,17) and to modify the metabolism of linoleic acid (18,19), serum total cholesterol and triglycerides and serum and tumor fatty acid profiles were also assessed.

MATERIALS AND METHODS

Tumor induction. Ninety inbred virgin female F344 rats, aged 28 days (Charles River Breeding Laboratories, North Wilmington, Massachusetts) were maintained on the standard NIH-07 diet (Zeigler Bros., Gardners, Pennsylvania) (20) until 50 days of age. All animals were then randomized into three groups of 30 animals each by recognized procedures (21) to equalize initial mean weights. On day 50 of age, all animals received a single dose of NMU (50 mg/kg body wt) by tail vein injection. The NMU (Ash Stevens Inc., Detroit, Michigan) was dissolved in a few drops of 3% acetic acid, and NMU was brought up to volume in distilled H_2O, yielding a stock solution of 10 mg/ml administered within 2 hr of formulation (22). Two days after carcinogen administration, animals were transferred to experimental diets and remained on them for the duration of the experiment.

At weekly intervals beginning four weeks after NMU injection, each rat was weighed, and the position and date of appearance of palpable tumors were recorded.

HF and low-fat (LF) diets. The adjusted HF diet used in these experiments is based on the recommendations of the Committee on Laboratory Animal Diets of the National Academy of Sciences (23-25), with slight modifications.

It has been found in our laboratory and those of others that rats adjust food intake so that similar energy intake is maintained, despite the fact that diets may differ substantially in energy density. Hence, animals will eat quantitatively less of a HF than LF diet. Consequently, unless the proportions of the other components in the diet are adjusted, animals fed a HF diet will take in substantially less protein, fiber, vitamins, minerals, etc., than

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those fed a LF diet. The adjustment recommended by the Committee on Laboratory Animal Diets was incorporated into our experimental protocol. The adjusted diet formulation ensured that animals fed a HF diet took in the same amount of vitamins, minerals and fiber as in the LF diet and that adequate antioxidant activity was present in the form of vitamin E, Se (as a cofactor of glutathione peroxidase) and vitamin A in each dietary group. The increase in fat in the HF diet was compensated for by a decrease in the amount of starch-dextrose (26) (Table 1). The HF and LF diets consisted of 23% and 5.0% corn oil. These percentages were designed to mimic the American (high risk) diet (40–45% of total calories as fat) and the Japanese (low risk) diet (16% of total calories as fat during the years 1957–1959) (2).

The LF diet was designed to provide ca. 5–6 cal/day of fat, based on an estimated consumption of 45–50 cal/day, (27) or ca. 12% of total. The HF diet, on the other hand, provided ca. 20–21 cal/day as fat, or 45% of total calories. All dietary ingredients were obtained from Dyets Inc. (Bethlehem, Pennsylvania) and were mixed in-house in our diet kitchen. Diets were formulated in 4-kg lots and stored in plastic bags at 4 C in the dark until used.

Animals were housed three to a cage in plastic cages covered with filter tops in a room controlled for temperature (24 C ± 2 C), light (12-hr cycle) and humidity (50%) and were administered diets (in powdered form) and tap water ad libitum.

Histopathology. Approximately 22 wk after NMU administration, all rats were killed by decapitation, and serum was collected following centrifugation of whole blood. Serum was stored at −20 C until assayed. Palpable tumors were excised and cut into two pieces—one of which was stored in liquid N_2 for fatty acid analysis and the other fixed in buffered formalin—blocked in paraffin and then sectioned and stained with hemotoxylin and eosin. Histological diagnosis of mammary tumors was based on the criteria outlined by Young and Hallowes (28).

Statistical evaluation of tumor data. Differences in tumor-free survival time among the three treatment groups were analyzed by the Kaplan-Meier Product Limit Method using a Fortran program provided by G. G. Gart and colleagues (29). Differences in tumor incidence were assessed by Fisher's Exact test (one-tail) and in tumor multiplicity by analysis of variance (ANOVA) after log transformation of the tumor count data. Overall, weight gains among the three treatment groups were compared by a two factor (diet and time) ANOVA with repeated measures (30).

Biochemistry. Nonfasting serum total cholesterol and triglycerides were determined by the use of a Gilford 3500 computer-directed auto analyzer by standard procedures (31,32).

Serum fatty acid analysis was carried out as described previously (4,33). Essentially, pooled serum samples (five/group) were extracted by a modified Radin (34) technique using hexane-isopropanol as the extraction solvent. The extracted lipid was then transmethylated using BF_3 (35), and the methyl esters of the fatty acids were separated by GLC. For tumor lipid analysis, frozen tumor tissue was pulverized under liquid N_2 in a mortar and pestle and then extracted in a similar manner as serum. The total lipid extract was then separated into neutral and phospholipid fractions using a silica gel column (4,36). The column was eluted first with chloroform (neutral lipid) and then a chloroform/methanol/water (65:26:4, v/v/v) mixture (phospholipid). The efficiency of extraction and separation was 94% for neutral lipids and 97% for phospholipids.

RESULTS

Tumor incidence, latency and multiplicity. Animals fed the MCT-containing diet exhibited a significantly lower total mammary tumor incidence when compared to animals fed a HF:corn oil diet (60% vs 87%, $p < 0.03$) (Table 2). However, when palpable adenocarcinomas alone were counted, the difference in incidence failed to attain statistical significance (57% vs 77%, $p < 0.08$). Likewise, when HF corn oil and LF corn oil-fed animals were compared in terms of total palpable mammary tumors, statistical significance was barely missed (66% vs 87%, $p < 0.06$); however, when only palpable adenocarcinomas

TABLE 1

Composition of Defined, Semipurified Diets (AIM-76A)

	Low fat diet	Adjusted high fat diets	
Ingredient	Corn oil[a] (g)	Corn oil[a] (g)	MCT[a] (g)
Casein	20.0	23.5	23.5
Cornstarch	52	32.9	32.9
Dextrose	13	8.30	8.30
Fat			
Corn oil	5	23.52	5.88
Medium chain triglyceride			17.64
DL-Methionine	0.3	0.35	0.35
Choline bitartrate	0.2	0.24	0.24
Alphacel	5	5.9	5.9
(AIN-76) Vitamin mix	1.0	1.18	1.18
(AIN-76) Mineral mix	3.5	4.11	4.11
Total	100.0	100.00	100.00
Energy value (cal/g)	3.89	4.73	4.73

[a]Five percent corn oil is added to assure adequate amounts of essential fatty acids.

were counted, differences in tumor incidence did not reach the level of statistical significance (60% vs 77%, $p > 0.10$).

Analysis of time-to-first-tumor curves (Fig. 1) indicated clearly that mammary tumors appeared more rapidly in the HF corn oil group than in the HF-MCT and LF-corn oil groups. The stepwise survival curves exhibited by the latter two groups were indistinguishable, whereas there was a significant delay in tumor appearance in these groups when compared to the HF-corn oil curve. The median times to first tumor in the HF-corn oil, HF-MCT and LF-corn oil groups were 90, 120 and 120, respectively (Table 3). Tumor multiplicity was similar in each group (Table 4), and no difference in tumor size was seen in the different treatment groups (data not shown).

Biochemistry. With regard to serum lipid concentrations, mean nonfasting cholesterol levels were significantly lower in animals fed 23% corn oil compared to those fed either 5% corn oil or the corn oil–MCT diet. No significant differences were found in serum triglycerides for any of the three treatment groups (Table 5).

TABLE 2

Influence of Medium Chain Triglycerides on the Incidence of NMU-Induced Mammary Tumors[a]

Dietary fat	% Fat	N[b]	Adenocarcinoma	Fibroadenoma	Total tumors
Corn	23	30	77[c] (23/30)[d]	10[c] (3/30)[d]	87[c] (26/30)[d]
Corn	5	30	60 (18/30)	6 (2/30)	66 (20/30)
Corn	6	30	57 (17/30)	3 (1/30)	60 (18/30)
+ Medium chain triglyceride	18				

All adenocarcinoma comparisons NS. Total tumors: 23% corn vs 5% corn = .062; 23% corn vs corn + MCT = 0.03; 5% corn vs corn + MCT = NS.
[a]Palpable tumors only.
[b]No. animals at risk.
[c]Tumor incidence (%).
[d]Number of tumor-bearing animals/number of animals at risk.

TABLE 3

Influence of Medium Chain Triglycerides on the Latent Period of NMU-Induced Mammary Tumors

Dietary fat	% Fat	N[a]	Mean latent period (days postinduction)	Median latent period (days)
Corn	23	30	86 ± 23[b]	90
Corn	5	30	117 ± 36	120
Corn	6	30	122 ± 40	120
+ Medium chain triglyceride	18			

[a]Number of animals at risk.
[b]Mean days to first tumor/±SD.

TABLE 4

Influence of Medium Chain Triglycerides on the Latent Period of NMU-Induced Mammary Tumors

Dietary fat	% Fat	No. adenocarcinoma/total animals with 1 or more adenocarcinomas
Corn	23	0.48 ± 48[a,c] (1.61)[c]
Corn	5	0.43 ± .56 (1.53)
Corn	6	0.58 ± 61 (1.78)
+ Medium chain triglyceride	18	

[a]Least square mean ($\log_e$)±SD.
[b]All pairwise comparisons NS (by one-way analysis of variance).
[c]Anti-$\log_e$.

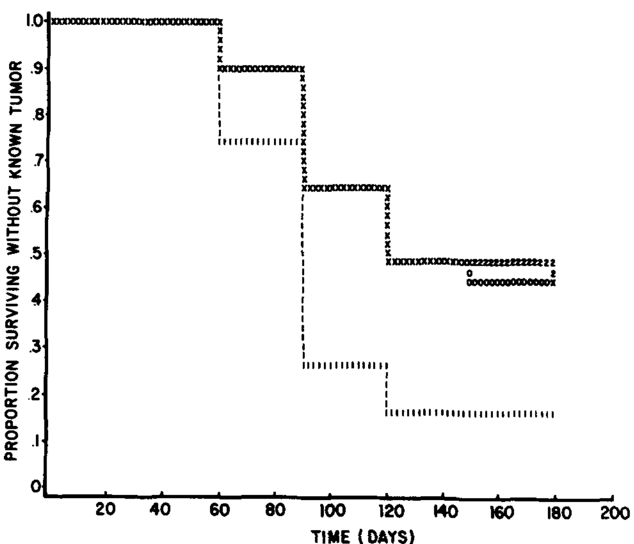

FIG. 1. Kaplan-Meier life tables curves for cumulative mammary tumor incidence. 0, 18% MCT + 6% corn oil; 1, 23% corn oil; 2, 5% corn oil; X, overlapping lines. Tests for overall trend: see ref. 9. Cox's test for adjusted trends, $p < 0.0035$. Pairwise comparisons: Cox's Test (conservative). 23% corn vs 5% corn, $p < 0.0099$; 23% corn vs MCT, $p < 0.0086$); 5% corn vs MCT, $p < 0.9$.

The fatty acid profiles of serum lipids differed primarily in the quantity of linoleic acid (18:2) present. Animals fed 23% corn oil diets exhibited twice the amount of LA as those fed 5% corn oil or MCT-containing diets. Differences in 14:0, 16:0, 16:1, 18:3 and 20:4 fatty acids were also noted (Table 6), though these were of a lower degree of magnitude.

Comparison of the fatty acid profiles in tumor neutral lipids (Table 7) indicated that the spectrum of fatty acids closely reflected that of the diet, particularly with regard to the essential fatty acid linoleic acid (18:2n-6); 10:0 and 12:0 fatty acids were detected only in the MCT group and at very low levels.

Comparison of phospholipid fatty acid profiles (Table 8) in NMU-induced tumors revealed a marked difference compared to neutral lipid profiles. In general, AA levels were higher than LA levels in the phospholipid fraction. When the phospholipid profiles of the three treatment groups were compared, it could be seen that LA still exhibited higher levels compared to the LF-corn oil and the HF-MCT groups. No differences were seen in AA levels, which tended to be highly variable both within and between groups.

Animal weight gains were similar in each group (Table 9), indicating that differences in type or quantity of

TABLE 5

Influence of Various Dietary Fats on Serum Lipid Concentrations

Dietary fat	% Fat	N[a]	Cholesterol	Triglycerides
Corn	23	30	80 ± 16[b,e] (81)[c] (53–137)[d]	117 ± 43[f] (116) (38–199)
Corn	5	30	108 ± 15 (107) (81–146)	112 ± 46 (98) (63–303)
Corn + Medium chain triglyceride	6 18	30	107 ± 15 (109) (66–131)	142 ± 83 (117) (65–420)

[a]Number of animals at risk.
[b]Arithmetic mean ± SD (mg/100 ml).
[c]Median.
[d]Range.
[e]Corn (23) vs MCT $P < .0001$. Corn (23) vs corn (5) $P < .0001$.
[f]All pairwise comparisons NS (by one-way analysis of variance).

TABLE 6

Comparison of Serum Lipid Fatty Acid Profiles in Animals Fed Diets Varying in Type and Amount of Fat

	Fatty acid percentages									
Fat (%)	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Corn (23)	—[a]	—	—	18.4[b] (±0.9)	—	19.1 (±1.5)	9.8 (±1.2)	28.5 (±1.7)	—	23.5 (±2.2)
Corn (5)	—	—	0.5 (±0.1)	22.9 (±0.7)	1.2 (±0.2)	17.6 (±3.9)	15.1 (±1.8)	13.2 (±1.3)	0.5 (±0.3)	29.1 (±4.4)
Corn (6) + MCT (18)	—	—	0.7 (±0.2)	24.5 (±5.0)	1.1 (±0.3)	19.4 (±1.4)	11.4 (±2.6)	13.3 (±2.7)	—	28.2 (±0.9)

[a]—, Not detectable.
[b]Percentage of total fatty acids; mean ± SD (five pooled samples/group).

TABLE 7

Tumor Neutral Lipid Fatty Acid Profile[a]

	Fatty acid percentages									
Fat (%)	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Corn (23) (n = 9)	—	—	1.4[c] (±0.79)	19 (±2.2)	1 (±0.26)	4.6 (±1.2)	28 (±1.5)	43 (±6.5)	1.0 (±0.9)	2.3 (±2.2)
Corn (5) (n = 8)			2 (±0.58)	26 (±1.9)	4.8 (±1.9)	3.7 (±1.4)	33 (±3.9)	28 (±4.7)		1.5 (±2.2)
Corn (6) + MCT (18) (n = 11)	1.0 (±0.7)	0.4 (±0.1)	2.5 (±.79)	29 (±2.8)	3.0 (±0.8)	7 (±2.8)	34 (±2.9)	19 (±6.3)	0.8 (±.67)	5.3 (±4.8)

[a]Percentage of total fatty acids.
[b]—, Not detectable.
[c]Mean ± SD.

TABLE 8

Tumor Phospholipid Fatty Acid Profile

	Fatty acid percentages									
Fat (%)	10:0	12:0	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Corn (23) (n = 8)	—[a]		1.5 (±2.2)	34[b] (±2.2)	1.0 (±0.1)	18 (±6.7)	19 (±4.8)	11[c] (±7.4)	—	17 (±9)
Corn (5) (n = 10)			1.0 (±0.6)	33 (±2.8)	1.3 (±0.6)	16 (±1.8)	21 (±3.9)	4 (±1.7)	—	24 (±6.5)
Corn (6) + MCT (18 (n = 10)		—	1.2 (±0.6)	33 (±8.2)	2 (±0.9)	16 (±5.7)	26 (±5.6)	5 (±2.4)	1 (±0.6)	17 (±8.6)

[a]—, Not detectable.
[b]Percentage of total fatty acids; mean ± SD.
[c]LA content: corn (23) vs corn (5), $p < 0.02$; corn (23) vs MCT, $p < 0.034$; corn (5) vs MCT, NS (all by two-tailed t-test).

TABLE 9

Cumulative Mean Animal Weights (g)

Weight (weeks postinduction)	Experimental group		
	Corn (23%)	Corn (18%) + MCT (6%)	Corn (5%)
0	100 ± 4	100 ± 4	101 ± 3
4	137 ± 6[a]	137 ± 7	135 ± 6
8	163 ± 9	161 ± 9	159 ± 7
12	169 ± 10	166 ± 10	165 ± 6
16	175 ± 10	176 ± 10	171 ± 8
20	176 ± 10	178 ± 12	175 ± 9
22	178 ± 11	178 ± 14	176 ± 10

Not significant by analysis of variance.
[a]Arithmetic mean ± SD; all comparisons of weight curves.

dietary fat did not alter food consumption patterns. Direct measurement of food consumption confirmed this finding: LF-fed animals consumed 11–12 g/day while HF-fed animals consumed 8–10 g/day.

DISCUSSION

This study indicates that a HF diet containing high levels of MCFA does not enhance the development of mammary tumors, in contrast to a HF diet containing the more common long chain fatty acids. Possible mechanisms underlying the observed effects of the MCT-containing diet may be either direct or indirect. A direct mechanism can be envisaged based on the unique physicochemical and biological properties of MCT. Although lipid in nature, MCT are absorbed and transported by the body in a manner more characteristic of carbohydrates than lipids. In contrast to long chain fatty acids, MCT do not enter lymph or chylomicrons, are not incorporated into membranes and are rapidly oxidized by the mitochondria via a carnitine-independent, rather than a carnitine-dependent, mechanism (16,17,37). The presence of only small amounts of MCFA in serum or tumor lipids of MCT-fed animals indicates that MCFA are rapidly absorbed and oxidized rather than stored in tissues. Hence, because of the unique physiological properties of MCT, the HF-MCT diet may exert biological effects more like those of a LF than a HF diet.

MCT may also act via indirect mechanisms involving actions at hormone receptors and/or essential fatty acid metabolism. With regard to the former, it has been shown by Knazek et al. (38) and Cave and Erickson-Lucas (39) that feeding MCT to rats lowered the number of prolactin receptors in both hepatic tissue and mammary tumors. Since prolactin is a recognized promoting substance in mammary tumorigenesis (40,41), its tumor growth-promoting effects could be attenuated by limiting, via dietary means, the number of receptors in the target organ available for activation by circulating prolactin. With regard to the latter, there have been several reports that MCT influence essential fatty acid metabolism (18,19). They have been shown, for example, to exert a sparing effect on the LA requirement for relief of the symptoms of essential fatty acid deficiency. These findings are of particular interest since essential fatty acids and their metabolism to prostaglandins appear to play a role in both the HF effect and the essential fatty acid deficiency syndrome (10,42,43–45).

Differences in serum and tumor fatty acid profiles in the three treatment groups were seen mainly in the LA content, which was significantly elevated in the HF-corn oil group. Of all the major fatty acids, only LA was elevated in the 23% corn oil animals. This fact is probably a reflection of its status as an essential fatty acid—that is, it cannot be synthesized de novo or from dietary precursors by the body, and therefore must be performed in the diet (43). Since Δ^6-desaturase (the rate-limiting enzyme in the pathway from LA to arachidonic acid [AA] and ultimately the whole range of prostaglandins), is subject to inhibition by long chain fatty acids (46), consumption of a HF-corn oil diet may suppress the activities of the fatty acid desaturases and/or chain-elongating enzymes and thereby slow the conversion of LA to its various metabolites. However, in the absence of any direct data on the fatty acid desaturases, one can only speculate on the metabolic basis for the observed differences in fatty acid patterns reported in the present study.

The fact that AA levels in tumor phospholipids (the primary precursor for intracellular prostaglandins) were similar in all three groups, despite the observed differences in tumor yield, casts doubt on the possibility that mammary tumor promotion may be regulated by the amount of AA available in membrane phospholipids for conversion to prostaglandins (43,47,48), and also on the possibility that dietary MCT modulate the metabolism of LA to AA in mammary tumors.

The intake of MCT has been associated with reductions in tissue and serum cholesterol (16). In this study, animals receiving the MCT-containing HF diet exhibited significantly higher, rather than lower, serum cholesterol compared to animals receiving 23% corn oil. The reason for this is uncertain. It may be because MCT limit cholesterol deposition in tissues (4) and the observed high serum cholesterol levels may therefore be due to a reapportionment of cholesterol from the tissue to the serum compartments. The suppression of serum cholesterol levels by diets high in polyunsaturated fat, such as corn oil, is a well-established phenomenon, although the mechanism by which this occurs is uncertain (49,50).

In conclusion, this study emphasizes the importance of viewing dietary lipids not only in terms of their physicochemical characteristics, i.e., chain length, degree of saturation and levels of isomerization, but also in terms of their specific physiological roles in body metabolism. In this regard, MCT, which are unique among triglycerides in their mode of absorption and transport, should provide a valuable tool for further exploration of the role of dietary fat in breast cancer development.

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ERRATUM

Avato et al., *Lipids 22,* 11 (1987), have requested a correction to the title of their paper as follows:

Epicuticular Waxes of Maize as Affected by the Interaction of Mutant *gl8* with *gl2*, *gl3*, *gl4* and *gl15*

Chiral Discrimination in the Exchange of α-Tocopherol Stereoisomers Between Plasma and Red Blood Cells

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The transport of 2*R*,4′*R*,8′*R*-α-tocopherol and 2*S*,4′*R*,8′*R*-α-tocopherol from plasma into rat red blood cell membranes occurs with essentially no chiral discrimination. The previously demonstrated (10) preference of red blood cell membranes favoring 2*R*,4′*R*,8′*R*-α-tocopherol over the 2*S*,4′*R*,8′*R* stereoisomer is shown to be due to better retention of the former compound, i.e., to preferential retention of natural vitamin E.
Lipids 22, 469–473 (1987).

It is generally accepted that vitamin E owes its bioactivity to its ability to inhibit lipid peroxidation in vivo (1). The major component of natural vitamin E, 2*R*,4′*R*,8′*R*-α-tocopherol (*RRR*-α-T), has long been known to show a greater activity in various animal bioassays than synthetic 2*RS*,4′*RS*,8′*RS*-α-tocopherol (*all-rac*-α-T) (1). Each of the eight diastereoisomers of α-T has been shown to have a different activity in the rat fetal gestation-resorption assay, with the natural stereoisomer, *RRR*-α-T, being the most active (2). However, the range in bioactivities is not large. Thus, although the major differences in the biopotencies of the α-T stereoisomers appear to originate in the chirality of carbon atom 2 (3–8), 2*S*,4′*R*,8′*R*-α-tocopherol (*SRR*-α-T) still has ca. 30% of the activity of *RRR*-α-T (2).

There appear to have been no real attempts to explain or explore the origin of the different bioactivities of the α-T stereoisomers. To us, the magnitude of the *RRR*-α-T/*SRR*-α-T difference appeared far too small to be due to some enzyme- or protein-mediated chemical or physical (e.g., transport) process (9). We therefore undertook and have recently reported the results of an in vivo "competitive" experiment involving these two stereoisomers (10). Three-week-old male rats were raised on a standard vitamin E–free diet to which we had added 36 mg of *RRR*-α-T acetate per kg of chow. After four weeks, the diet was changed to one based on the same chow but with the natural α-T acetate replaced by 18 mg 5,7-$(CD_3)_2$-*RRR*-α-T + 18 mg 5-CD_3-*SRR*-α-T acetates per kg of chow. Analyses of blood and tissue samples taken at various times yielded the ratio d_6-*RRR*-α-T/d_3-*SRR*-α-T, which gives a measure of the discrimination in favor of the natural isomer, *RRR*-α-T/*SRR*-α-T, in the sample. One of the more interesting results from this study related to the degree of chiral selectivity in blood plasma and red blood cells (RBC). Both the plasma and the RBC discriminated in favor of natural α-T, and the *RRR*-α-T/*SRR*-α-T ratio increased with time. However, the RBC membranes were more selective than the plasma lipoproteins at all times: the ratio, $[(RRR\text{-}\alpha\text{-T})/(SRR\text{-}\alpha\text{-T})]_{RBC}/[(RRR\text{-}\alpha\text{-T})/(SRR\text{-}\alpha\text{-T})]_{plasma}$, was equal to 1.35 ± 0.13 throughout the five months of the experiment (10). The observed greater chiral selectivity of RBC membranes compared with plasma might arise from a variety of kinetic phenomena, including (i) *RRR*-α-T is preferentially transferred from plasma to RBC; (ii) *SRR*-α-T is preferentially transferred from RBC to plasma; and (iii) both (i) and (ii) occur. In this paper, we report the results of experiments designed to distinguish among these three possibilities.

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MATERIALS AND METHODS

Materials. The syntheses of the deuterium-labeled α-tocopherols have been described previously (10).

Methods. Three groups of experiments were conducted. In all experiments 3-wk-old male Sprague-Dawley rats bred at the NRCC specific pathogen-free facility were weaned and placed on a standard AIN-76 diet [major components: sucrose, 50%; casein, 20%; corn starch, 15%; tocopherol-stripped corn oil, 5% (11)] that contained extra menadione [10 times the level in the original diet (12,13)] and 36 mg α-T acetate (α-T-Ac) per kg of diet. The control diet contained 36 mg of unlabeled *RRR*-α-T-Ac per kg (diet 0). For the first experiment, a diet was used that contained 36 mg of 5-CD_3-*RRR*-α-T-Ac (d_3-*RRR*-α-T-Ac) per kg (diet 1). For the second experiment, a diet was used that contained 18 mg of 5,7-$(CD_3)_2$-*RRR*-α-T-Ac (d_6-*RRR*-α-T-Ac) plus 18 mg of 5-CD_3-*SRR*-α-T-Ac (d_3-*SRR*-α-T-Ac) per kg (diet 2). For the third experiment, the diet contained 12 mg of d_6-*RRR*-α-T-Ac plus 24 mg of d_3-*SRR*-α-T-Ac per kg (diet 3).

Typically, one rat raised on the control diet (diet 0) and one rat of identical age raised on one of the deuterium-labeled α-T-Ac diets were used in each experiment. Blood (~10 ml), obtained from nonfasted animals by heart puncture under halothane anesthesia, was collected over 200 μl of phosphate buffer containing disodium ethylenediaminetetraacetate (10 mg) and immediately placed in a bucket of ice. Equal volumes of blood from the two animals were placed in centrifuge tubes and spun at 12,000 × g for 10 min at 4 C. The plasma was separated and to it was added 1.67 mg glucose/ml plasma (an amount approximately equivalent to 1 mg/ml of reconstituted blood). The red blood cells were washed by resuspension in ice-cold phosphate-buffered saline (5 mM, pH 7.4), spinning at 270 × g for 10 min, removal of the supernatant and repetition of the whole procedure twice. At the end of the last wash, the red blood cells were packed by centrifugation at 12,000 × g for 10 min. The temperature of the plasma and packed RBC was then raised to 37 C by shaking in a water bath for 15 min. The RBC from one rat were then mixed with the plasma from the other rat (total volume ca. 10 ml; hematocrit 37 ± 1%) with the temperature being maintained at 37 C. Samples (1 ml each) were withdrawn at intervals and centrifuged, the plasma (~0.4 ml) was collected and the RBC were washed three times with phosphate-buffered saline as described above (the period of centrifugation after each wash was reduced to 5 min). Lipid was extracted from the plasma by the usual ethanol/heptane procedure (10,14,15) and from the RBC by the sodium dodecyl sulfate (SDS) method (10,15). The direct extraction of lipid

from the RBC was performed by first adding sodium ascorbate (ca. 100 mg) to the packed RBC followed by SDS (0.1 M, 4 ml), ethanol (5 ml) and *n*-heptane (2 ml). The heptane extract was passed through an analytical high performance liquid chromatograph (15), and the eluent containing the α-T fraction was collected. The α-Ts were then converted to their trimethylsilyl ether derivatives prior to analyses for d_0-, d_3- and d_6-α-T by gas chromatography/mass spectrometry. These steps were performed as described previously (10).

We note that total concentrations of the α-Ts in the plasma and RBC used in an experiment are not required in the calculation of the discrimination factors for the relative rates of transfer of *RRR*-α-T vs *SRR*-α-T (see below).

RESULTS

Our initial experiment on α-T exchange was chosen to compare our procedure using deuterium-labeled α-T with literature data of Bjornson et al. (16), who used a tritium-labeled α-T. Plasma and RBC from a rat fed for 64 days after weaning on diet 1 (d_3-*RRR*-α-T-Ac) were mixed with RBC and plasma, respectively, from a rat fed diet 0 (d_0-*RRR*-α-T-Ac) for the same length of time. Following the procedure of Bjornson et al. (16), we plotted experimental values of log $\{([d_3]_t-[d_3]_\infty)/([d_3]_0-[d_3]_\infty)\}$ against time, where $[d_3]$ represents the concentration of d_3-*RRR*-α-T and the subscripts t, 0 and ∞ refer to times t, the start of the experiment and the time at which equilibrium had been reached (ca. 12 hr), respectively. The concentration of d_3-*RRR*-α-T was measured in both RBC and plasma. The RBC contain a smaller "pool" of α-T than the plasma (ca. 30–50% of the plasma "pool" in blood from fasted rats (17,18) but typically ca. 20% in the reconstituted blood from the nonfasted rats used in our experiments). There was therefore a correspondingly greater change in the relative concentration of RBC d_3-*RRR*-α-T than plasma d_3-*RRR*-α-T, and thus the RBC yielded more reliable kinetic data. For the transfer of d_3-*RRR*-α-T from labeled plasma into unlabeled RBC, the semilog plot for d_3 uptake by RBC had a slope of 0.31 hr^{-1} (loss from plasma gave a slope of 0.25 hr^{-1}). The RBC slope yields a half-life for exchange (toward equilibrium) of *RRR*-α-T between plasma and RBC, $\tau_{1/2}$, of 0.69/0.31 = 2.2 hr. These results are in perfect agreement with those of Bjornson et al. (16) for their plasma → RBC experiment, namely, slope = 0.31 hr^{-1}, $\tau_{1/2}$ = 2.2 hr. Similarly, for the transfer of d_3-*RRR*-α-T from labeled RBC into unlabeled plasma, the semilog plot of d_3 loss by the cells had a slope of 0.28 hr^{-1} (uptake by plasma gave a slope of 0.20 hr^{-1}) corresponding to $\tau_{1/2}$ = 2.5 hr. For their RBC → plasma experiment, Bjornson et al. (16) give slope = 0.32 hr^{-1}, $\tau_{1/2}$ = 2.2 hr. The excellent agreement between our results and theirs gave us confidence in the reliability of our experimental procedures.

For the competitive experiments involving d_6-*RRR*-α-T and d_3-*SRR*-α-T, we measured only the initial rates of uptake (or loss) of these two tocopherols (i.e., for the first 5–10% at the overall approach to equilibrium). This made it possible to determine separately the stereoisomeric discrimination occurring upon transfer of α-T from plasma to RBC and vice-versa (see Appendix). Typically, measurements of the relative concentrations of d_6-*RRR*-α-T and d_3-*SRR*-α-T (and, of course, d_0-*RRR*-α-T) in the plasma and RBC were taken every 10 min for 1 to 1.5 hr. Because of the preference of blood for the natural stereoisomer of α-T (10), there was about twice as much d_6-*RRR*-α-T as d_3-*SRR*-α-T in the plasma and RBC of the rats raised on diet 2 ([d_6-*RRR*-α-T-Ac]/[d_3-*SRR*-α-T-Ac] = 1.0). However, for the rats on diet 3 ([d_6-*RRR*-α-T-Ac]/[d_3-*SRR*-α-T-Ac] = 0.5), the plasma and RBC contained quantities of these two stereoisomers that were more nearly equal. For example, a rat raised for 38 days on diet 3 had [d_6-*RRR*-α-T]/[d_3-*SRR*-α-T] ratios of 0.96 and 1.49 in its plasma and RBC, respectively. [The differential selectivity favoring the natural stereoisomer in the RBC, i.e., 1.49/0.96 = 1.55 is within the range found previously for rats consuming diet 2 (10).] The percentage concentrations of d_6-*RRR*-α-T and d_3-*SRR*-α-T in plasma and RBC following the mixing of plasma and RBC from this rat with the RBC and plasma, respectively, from a rat raised for 38 days on diet 0 (d_0-*RRR*-α-T-Ac) are given in Table 1. These data are quite typical.

TABLE 1

Concentrations of d_6-*RRR*-α-T and d_3-*SRR*-α-T as a Percentage of the Total α-T in Plasma and RBC Before and at Various Times After Mixing Plasma and RBC from a Rat Raised 38 Days on Diet 3 and a Rat Raised 38 Days on Diet 0

	Time (min)										
	0	10	20	30	40	50	60	70	80	90	100
(d_6 + d_3)-RBC + d_0-plasma											
d_6-*RRR*-α-T in RBC	55.2	52.3	49.0	46.3	43.8	41.7	39.4	37.4	35.9	33.7	30.8
d_3-*SRR*-α-T in RBC	37.0	34.2	31.6	29.3	27.2	25.0	23.1	21.9	20.4	18.9	16.4
$(D^{RBC \to P})^{RBC}$		0.71	0.77	0.78	0.78	0.76	0.76	0.79	0.78	0.80	0.79
d_6-*RRR*-α-T in plasma	0	0.33	0.71	1.06	1.38	1.77	2.11	2.40	2.68	3.20	3.35
d_3-*SRR*-α-T in plasma	0	0.25	0.62	0.95	1.21	1.58	1.90	2.15	2.36	2.76	2.90
$(D^{RBC \to P})^{P}$		0.89	0.77	0.78	0.74	0.75	0.74	0.75	0.77	0.78	0.78
(d_6 + d_3)-Plasma + d_0-RBC											
d_6-*RRR*-α-T in RBC	0	1.15	2.47	3.08	4.07	5.08	5.88	6.60	7.50	8.65	9.51
d_3-*SRR*-α-T in RBC	0	0.98	2.62	2.89	4.02	5.00	5.33	5.98	6.47	7.31	7.99
$(D^{P \to RBC})^{RBC}$		1.22	0.98	1.11	1.05	1.06	1.15	1.15	1.21	1.23	1.24
d_6-*RRR*-α-T in plasma	45.8	45.3	44.5	43.9	43.5	42.8	42.3	41.6	41.3	40.6	40.4
d_3-*SRR*-α-T in plasma	47.7	47.0	46.5	45.8	45.2	44.8	44.3	43.9	43.4	42.9	42.6
$(D^{P \to RBC})^{P}$		0.82	1.08	1.01	0.93	1.07	1.06	1.13	1.08	1.11	1.11

RRR-α-T, 2*R*,4′*R*,8′*R*-α-tocopherol; *SRR*-α-T, 2*S*,4′*R*,8′*R*-α-tocopherol; RBC, red blood cells.

Let us consider first RBC containing d_6-*RRR*-α-T and d_3-*SRR*-α-T (plus some d_0-*RRR*-α-T), which are mixed with plasma containing only d_0-*RRR*-α-T. We define a quantity, $D^{RBC \to P}$, that represents the time-independent degree of discrimination (if any) in favor of transport of d_6-*RRR*-α-T over that of transport of d_3-*SRR*-α-T from the RBC to the plasma (RBC→P). If we are monitoring the loss of the two stereoisomers from the RBC, we can write (see Appendix):

$$\left[\frac{(RRR)_0 - (RRR)_t}{(SRR)_0 - (SRR)_t}\right]^{RBC} \left[\frac{(SRR)_0}{(RRR)_0}\right]^{RBC} = (D^{RBC \to P})^{RBC} \qquad [1]$$

In this equation, *RRR* and *SRR* refer to percentage concentrations of d_6-*RRR*-α-T and d_3-*SRR*-α-T, respectively, subscripts refer to the time of the measurement (0 = start of the experiment) and the superscript RBC reminds us that these are measurements on RBC. (Initial concentrations can be used because the run is carried on to only 5 or 10% of final equilibration.) Values of $(D^{RBC \to P})^{RBC}$ calculated from each individual measurement are given in Table 1.

On the other hand, if we are monitoring the gain of the two stereoisomers by the plasma, we write:

$$\left[\frac{(RRR)_t}{(SRR)_t}\right]^{P} \left[\frac{(SRR)^0}{(RRR)_0}\right]^{RBC} = (D^{RBC \to P})^{P} \qquad [2]$$

where the superscript P reminds us that these are measurements on the plasma. Individual values of $(D^{RBC \to P})^{P}$ are given in Table 1. The mean values of $(D^{RBC \to P})^{RBC}$ and $(D^{RBC \to P})^{P}$ should, of course, be equal. For the RBC to plasma transfer experiments the agreement between these two independently derived quantities is generally rather good (see Table 2), because it is relatively easy to measure the gain of the two stereoisomers by the plasma since their initial concentration in the plasma is 0. The measurement of $(D^{RBC \to P})^{RBC}$ is also fairly reliable because the total "pool" of α-T in RBC (in the reconstituted blood) is lower than in the plasma (17,18). The decrease in the quantities of deuterated α-tocopherols can therefore be determined fairly reliably.

For the companion pairs of experiments in which plasma containing the deuterated α-tocopherols was mixed with RBC containing only d_0-*RRR*-α-T, we can define a quantity, $D^{P \to RBC}$, that represents the time independent degree of discrimination (if any) in favor of transport of d_6-*RRR*-α-T over that of transport of d_3-*SRR*-α-T from plasma to RBC. If we are monitoring the gain of the two stereoisomers by RBC, we write:

$$\left[\frac{(RRR)_t}{(SRR)_t}\right]^{RBC} \left[\frac{(SRR)_0}{(RRR)_0}\right]^{P} = (D^{P \to RBC})^{RBC} \qquad [3]$$

while for the loss of the stereoisomers by the plasma, we write:

$$\left[\frac{(RRR)_0 - (RRR)_t}{(SRR)_0 - (SRR)_t}\right]^{P} \left[\frac{(SRR)_0}{(RRR)_0}\right]^{P} = (D^{P \to RBC})^{P} \qquad [4]$$

Values for these two quantities for each individual measurement are given in Table 1.

Once again, the mean values for $(D^{P \to RBC})^{RBC}$ and $(D^{P \to RBC})^{P}$ should be equal. However, in this type of experiment, generally only $(D^{P \to RBC})^{RBC}$ can be fairly reliably determined. There is relatively little change in the concentrations of

TABLE 2

Summary of Initial α-T Isotopic Compositions as a Percentage of the Total α-T in the Labeled Blood Fraction and of Calculated Discrimination Factors Favoring Transport of d_6-*RRR*-α-T over Transport of d_3-*SRR*-α-T from RBC to Plasma and from Plasma to RBC

	Diet number					
	2	2	2	3	3	3
Days on diet	101	121	129	22	38	64
(d_6 + d_3)-RBC + d_0-plasma						
d_6-$(RRR)_0$ in RBC (%)	65.8	75.0	70.9	48.8	55.2	54.4
d_3-$(SSR)_0$ in RBC (%)	19.3	23.1	22.0	39.8	37.0	30.1
$[d_6\text{-}(RRR)_0/d_3\text{-}(SRR)_0]^{RBC}$	3.42	3.26	3.22	1.23	1.49	1.81
No. of data points	4	5	6	7	10	8
$(D^{RBC \to P})^{RBC}$ ± S.D.[a]	0.87 ± 0.09	0.79 ± 0.03	0.83 ± 0.09	0.71 ± 0.05	0.77 ± 0.02	0.73 ± 0.09
$(D^{RBC \to P})^{P}$ ± S.D.[a]	0.76 ± 0.08	0.87 ± 0.02	0.76 ± 0.03	0.71 ± 0.02	0.78 ± 0.04	0.77 ± 0.04
(d_6 + d_3)-plasma + d_0-RBC						
d_6-$(RRR)_0$ in plasma (%)	67.4	70.5	67.5	41.4	45.8	52.1
d_3-$(SRR)_0$ in plasma (%)	28.6	27.9	31.0	50.5	47.7	42.6
$[d_6\text{-}(RRR)_0/d_3\text{-}(SRR)_0]^{P}$	2.35	2.53	2.18	0.82	0.96	1.22
No. of data points	5	5	8	6	10	6
$(D^{P \to RBC})^{RBC}$ ± S.D.[a]	1.08 ± 0.09	0.72 ± 0.38	0.99 ± 0.07	1.11 ± 0.14	1.15 ± 0.06	1.16 ± 0.07
$(D^{P \to RBC})^{P}$ ± S.D.[a]	1.03 ± 0.09	1.05 ± 0.05	0.94 ± 0.19	1.02 ± 0.15	1.04 ± 0.09	1.13 ± 0.11

α-T, α-tocopherol; RBC, red blood cells.

[a]Mean values for the six experiments ± S.E. of this mean are $(D^{RBC \to P})^{RBC}$ = 0.78 ± 0.05; $(D^{RBC \to P})^{P}$ = 0.78 ± 0.05; $(D^{P \to RBC})^{RBC}$ = 1.04 ± 0.15; $(D^{P \to RBC})^{P}$ = 1.04 ± 0.06.

d_6-*RRR*-α-T and d_3-*SRR*-α-T in the plasma, so the measurement of $(D^{P \to RBC})^P$ is usually subject to somewhat larger errors.

Results of our six d_6-*RRR*-α-T/d_3-*SRR*-α-T competitive kinetic experiments are summarized in Table 2.

DISCUSSION

As mentioned in the introduction, we have previously shown that in vivo the *RRR*-α-T/*SRR*-α-T ratio is higher in RBC than in the surrounding plasma (10). Our present results identify the major kinetic reason for the RBC biodiscrimination: RBC preferentially retain the natural stereoisomer. That is, *SRR*-α-T is transported from the RBC to the plasma more rapidly than *RRR*-α-T: $(D^{RBC \to P})^{RBC} = (D^{RBC \to P})^P = 0.78 \pm 0.05$ (mean of the six experimental results ± S.E. of mean). If there is any biodiscrimination in the transport from plasma to RBC, it appears to favor very slightly the transport of *RRR*-α-T: $(D^{P \to RBC})^{RBC} = 1.04 \pm 0.15$, $(D^{P \to RBC})^P = 1.04 \pm 0.06$ (mean of the six experimental results ± S.E. of mean, see Table 2). However, this preference for entry of *RRR*-α-T into RBC is well within our experimental error. Were biodiscrimination by RBC due solely to preferential retention of *RRR*-α-T, our earlier in vivo results would require that $D^{RBC \to P} = 0.74 \pm 0.07$. This value is consistent with our present direct measurement of this quantity, 0.78 ± 0.05.

The preferential retention of (natural) *RRR*-α-T by RBC must not be taken as providing supporting evidence for the existence of specific binding sites for *RRR*-α-T in these cells, as suggested by Kitabchi and Wimalasena (19). After all, the degree of biodiscrimination is rather small. We believe it to be of a magnitude consistent with the degree of chiral discrimination that might be exerted as a "solvent effect" by the chiral molecules that go to make up the RBC membrane (phospholipids, cholesterol, proteins, etc.). That is, the chirality "sensed" by tocopherol in the more structured environment of the RBC membrane is greater than in the lipid milieu of the low density lipoprotein. An attempt to check on this point by partitioning the deuterated tocopherols between a silanized glass surface (16,20) and RBC suspended in phosphate-buffered saline was unsuccessful. No transfer between the surface and the RBC could be observed.

Finally, there was no indication in the exchange experiments that the α-T located on the inside of the RBC exchanged more slowly than the α-T on the outside of the cell. Specifically, there was no indication of biphasic kinetics or of the existence of a pool of nonexchanging α-T. This implies that the half-life, $t_{1/2}$, for movement of α-T from the inside monolayer of the RBC membrane to the outside monolayer is less than 10^4 sec and probably is less than 10^3 sec. We have previously shown that motion of α-T between the inside and outside monolayers of unilamellar phospholipid vesicles is slow on the nuclear magnetic resonance time scale (21). Assuming α-T behaves similarly in RBC and liposomes, we conclude that 10^{-3} sec $< t_{1/2} \leqslant 10^3$ sec. The very broad limits on the rate of α-T "flip-flop" reflect the current state of knowledge concerning the dynamics of α-T in membranes.

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APPENDIX: DERIVATION OF THE DISCRIMINATION PARAMETER, D

When deuterium-labeled *RRR*- and *SRR*-stereoisomers are present initially either entirely in RBC or entirely in plasma, the initial rate of loss of each stereoisomer from the labeled blood component can be written as

$$\frac{d(RRR)}{dt} = -k_R(RRR)_0 \qquad [5]$$

$$\frac{d(SRR)}{dt} = -k_S\,(SRR)_0 \qquad [6]$$

where k_R and k_S are rate constants for loss of the *RRR*- and *SRR*-stereoisomers, respectively. The discrimination parameter, D, is defined as

$$D = \frac{k_R}{k_S} \qquad [7]$$

Substituting for k_R and k_S in [7],

$$D = \frac{\dfrac{d(RRR)}{dt}}{\dfrac{d(SRR)}{dt}} \cdot \frac{(SRR)_0}{(RRR)_0}$$

For a small time interval, Δt,

$$D \cong \frac{\dfrac{\Delta(RRR)}{\Delta t}}{\dfrac{\Delta(SRR)}{\Delta t}} \cdot \frac{(SRR)_0}{(RRR)_0}$$

$$\text{i.e.,}\quad D \cong \frac{(RRR)_0 - (RRR)_t}{(SRR)_0 - (SRR)_t} \cdot \frac{(SRR)_0}{(RRR)_0} \qquad [8]$$

Similarly, an equation can be written corresponding to the discrimination in the gain of the two stereoisomers by the unlabeled blood component.

Effects of Phorbol Esters, A23187 and Vasopressin on Oleate Metabolism in Isolated Rat Hepatocytes

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Studies were conducted to compare the metabolic effects of vasopressin, 4β-phorbol-12-myristate-13-acetate (PMA) and A23187 on ketogenesis and oleate metabolism in isolated hepatocytes from fed rats. Vasopressin inhibited the formation of acid-soluble products from [1-^{14}C]oleate (0.25 mM, 0.5 mM and 1 mM), the inhibition being most marked at low (0.25 mM) concentration of oleate. Conversion of [1-^{14}C]oleate into $^{14}CO_2$ and esterified products was stimulated by vasopressin. The stimulatory effect of this hormone on $^{14}CO_2$ production was most marked at high (1 mM) concentration of oleate, whereas that on [1-^{14}C]-oleate esterification was most marked at low (0.25 mM) concentration of oleate. These vasopressin actions were abolished when hepatocytes were incubated in the absence of calcium in the medium. Our results strongly suggest that both increase in esterification and increase in oxidation to CO_2 contribute to the anti-ketogenic action of vasopressin when oleate is added as substrate, although the relative extent of their contribution varies according to the oleate concentration. The anti-ketogenic action of vasopressin was mimicked by PMA but not by A23187. PMA also caused a stimulation of [1-^{14}C]oleate esterification although the effect was diminished at 1 mM [1-^{14}C]oleate. A23187 failed to affect [1-^{14}C]oleate esterification. The metabolic effects of PMA were elicited in the absence of extracellular calcium, too. Conversion of [1-^{14}C]oleate into $^{14}CO_2$ was only slightly increased by both PMA and A23187 when 1 mM [1-^{14}C]oleate was added as substrate. The marked stimulatory effect of vasopressin on $^{14}CO_2$ production from [1-^{14}C]oleate was not reproduced even by the combination of PMA and A23187. The possible involvement of protein kinase C and calcium mobilization in the regulation of oleate metabolism is discussed.
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Vasopressin has been demonstrated not to change the hepatic level of cyclic AMP (1) but to act through the hydrolysis of phosphatidylinositol 4,5-bis-phosphate (2–4). Indeed, evidence is accumulating that vasopressin increases inositol trisphosphate (5,6) as well as diacylglycerol (7–9) in isolated rat hepatocytes. Inositol trisphosphate is a second messenger for intracellular Ca^{++} mobilization (5,6,10,11), whereas diacylglycerol acts as a second messenger to activate calcium-activated, phospholipid-dependent protein kinase, protein kinase C (12,13). The calcium ionophore A23187 can cause Ca^{++} mobilization in hepatocytes (14). The tumor-promoting phorbol esters such as 4β-phorbol-12-myristate-13-acetate (PMA) can activate protein kinase C by substituting for diacylglycerol (13,15). These two compounds, therefore, appear to be good probes for investigating the possible role of a bifurcated transducing mechanism in the action of vasopressin.

Vasopressin affects oleate metabolism in isolated hepatocytes of fed rats, i.e., it inhibits ketogenesis from oleate (16,17), stimulates the conversion of [1-^{14}C]oleate into $^{14}CO_2$ (18) and increases [1-^{14}C]oleate esterification (16). However, no general consensus exists as to which transducing mechanism is responsible for each of these vasopressin actions. Furthermore, it remains controversial as to how these metabolic effects are interrelated. The present study was, therefore, designed to compare the effects of vasopressin, phorbol esters and A23187 on ketogenesis and oleate metabolism in isolated hepatocytes of fed rats and to gain further insight into the hormonal regulation of oleate metabolism.

MATERIALS AND METHODS

Animals. Male Wistar rats (300–400 g) were used. All animals were subjected to a 12-hr light/12-hr dark cycle, with the light period starting at 7:00 a.m., for at least 7 days prior to the experiment. The rats were allowed free access to water and standard laboratory food (Oriental Yeast Co., Tokyo, Japan).

Isolation and incubation of hepatocytes. Preparation of hepatocytes commenced between 9:00 and 10:00 a.m. by the method of Berry and Friend (19) with the modifications described by Harris (20). Calcium-depleted cells were prepared by omitting $CaCl_2$ from the Krebs-Henseleit buffer used for washing. They were incubated in Krebs-Henseleit buffer containing 1 mM ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid (EGTA) but no calcium. Phorbol esters and calcium ionophore A23187 were dissolved in dimethyl sulfoxide. The concentration of dimethyl sulfoxide in the incubation medium was always 1% (v/v). Incubations were carried out at 37 C for 30 min with 70–90 mg wet weight of hepatocytes in 25-ml Erlenmeyer flasks in a final volume of 2 ml of incubation medium and were terminated with $HClO_4$.

Analytical methods. Metabolite assays were conducted on KOH-neutralized $HClO_4$ extracts. The following metabolites were determined spectrophotometrically by standard enzymatic methods: glucose (21), lactate and pyruvate (22), and acetoacetate and β-hydroxybutyrate (23). Ketogenesis was expressed in terms of an accumulation of total ketone bodies (acetoacetate plus β-hydroxybutyrate). The measurements of $^{14}CO_2$ production and the formation of radioactive acid-soluble products (mainly ketone bodies) were performed essentially as described by Christiansen et al. (24) and Yount and Harris (25). The extent of [1-^{14}C]oleate esterification was determined as described by McCune et al. (26).

Materials. [1-^{14}C]Oleate was obtained from New England Nuclear (Boston, MA). Collagenase (Type II) was obtained from Worthington Biochemical Corp. (Freehold, NJ). [Arginine]vasopressin was purchased from Peptide Institute (Minoh, Japan). Phorbol esters were obtained from Sigma Chemical Co. (St. Louis, MO). A23187 was

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Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)N,N′-tetraacetic acid; PMA, 4β-phorbol-12-myristate-13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; 4β-PDD, 4β-phorbol-12,13-didecanoate.

from Calbiochem (San Diego, CA). Nucleotides, coenzymes and crystalline enzymes were obtained from Boehringer Mannheim (Mannheim, FRG) or from Sigma.

Statistical analysis. The results are expressed as means ± SEM. Statistical evaluation of the data was made by means of Student's t-test for paired data.

RESULTS

Effects of vasopressin, PMA and A23187 on glucose release, glycolysis and ketogenesis in the presence of 0.5 mM oleate. Metabolic effects of 10^{-7} M vasopressin, 10^{-6} M PMA and 10^{-5} M A23187 in the presence of 0.5 mM oleate are summarized in Table 1. Vasopressin stimulated glucose release and lactate and pyruvate accumulation in the presence of 0.5 mM oleate. The ratio of lactate/pyruvate, an index of the NAD^+ redox state in the cytoplasm, was significantly decreased (more oxidized) by vasopressin. Production of total ketone bodies was suppressed by this hormone. Along with the inhibition of ketogenesis, the ratio of β-hydroxybutyrate/acetoacetate, an index of the NAD^+ redox state in the mitochondria, was also decreased (more oxidized). The present results are in good agreement with the vasopressin actions previously observed in the presence of 1 mM oleate (16,17). A23187 produced a marked increase in glucose release like vasopressin, whereas PMA caused only a slight increase in it. Lactate and pyruvate accumulation was stimulated by both PMA and A23187, although the effect of the latter was more potent. A23187 significantly decreased the ratio of lactate/pyruvate, while PMA did not affect it. Ketogenesis from oleate was inhibited by PMA but not affected by A23187. Furthermore, PMA caused a decrease in the ratio of β-hydroxybutyrate/acetoacetate. The combination of PMA and A23187 produced an additive stimulation of lactate and pyruvate accumulation. Increase in glucose release by A23187 and inhibition of ketogenesis by PMA were not affected by the addition of PMA and A23187, respectively.

Effects of vasopressin, PMA and A23187 on glucose release, glycolysis and ketogenesis in the absence of oleate. Metabolic effects of 10^{-7} M vasopressin, 10^{-6} M PMA and 10^{-5} M A23187 in the absence of oleate are summarized in Table 2. The effect of vasopressin on glucose

TABLE 1

Effects of 4β-Phorbol-12-myristate-13-acetate (PMA), A23187 and Vasopressin on Glucose Release, Glycolysis and Ketogenesis in the Presence of 0.5 mM Oleate[a]

Metabolic parameter	Control	PMA (10^{-6} M)	A23187 (10^{-5} M)	PMA (10^{-6} M) + A23187 (10^{-5} M)	Vasopressin (10^{-7} M)
Glucose release[b]	55.9 ± 4.1	60.6 ± 4.5* (108)	69.4 ± 5.5* (124)	70.5 ± 5.7* (126)	70.7 ± 6.0* (126)
Lactate plus pyruvate[b]	8.0 ± 1.7	13.1 ± 2.9* (164)	18.6 ± 3.0* (233)	22.2 ± 3.8* (278)	15.2 ± 3.3* (190)
Lactate/pyruvate ratio	3.9 ± 0.3	3.5 ± 0.4 (90)	2.8 ± 0.1* (72)	2.6 ± 0.2* (67)	2.7 ± 0.1* (69)
Acetoacetate plus β-hydroxybutyrate[b]	10.0 ± 1.0	7.8 ± 0.9* (78)	10.1 ± 0.8 (101)	7.6 ± 0.8* (76)	6.8 ± 0.7* (68)
β-Hydroxybutyrate/acetoacetate ratio	0.73 ± 0.05	0.50 ± 0.03* (68)	0.64 ± 0.04 (88)	0.52 ± 0.04* (71)	0.58 ± 0.07* (79)

[a]Incubations were conducted for 30 min. Results are expressed as the mean ± SEM for five hepatocyte preparations from fed rats. Values in parentheses are percent of control. *, $P < 0.05$, compared with control incubations.

[b]Results are expressed as μmol/30 min/g wet wt.

TABLE 2

Effects of 4β-Phorbol-12-myristate-13-acetate (PMA), A23187 and Vasopressin on Glucose Release, Glycolysis and Ketogenesis in the Absence of Oleate[a]

Metabolic parameter	Control	PMA (10^{-6} M)	A23187 (10^{-5} M)	PMA (10^{-6} M) + A23187 (10^{-5} M)	Vasopressin (10^{-7} M)
Glucose release	51.1 ± 3.9	55.5 ± 4.4* (109)	67.2 ± 5.8* (132)	68.2 ± 8.5* (133)	65.5 ± 6.0* (128)
Lactate plus pyruvate	24.1 ± 2.8	28.9 ± 3.4* (120)	25.5 ± 2.8 (106)	29.4 ± 3.6* (122)	25.1 ± 2.6 (104)
Acetoacetate plus β-hydroxybutyrate	1.7 ± 0.1	1.6 ± 0.2 (94)	4.9 ± 0.5* (288)	4.5 ± 0.4* (265)	1.7 ± 0.2 (100)

[a]Incubations were conducted for 30 min. Results are expressed as the mean ± SEM for five hepatocyte preparations from fed rats (μmol/30 min/g wet wt). Values in parentheses are percent of control. *, $P < 0.05$, compared with control incubations.

release was mimicked by A23187. PMA slightly increased glucose release. Corvera and García-Sáinz (27) reported that A23187 but not PMA stimulated glucose release in isolated hepatocytes of fed rats. The slight increase in glucose release by PMA observed in the present study may reflect the inactivation of glycogen synthase by PMA (28,29). Vasopressin and A23187 exerted no effect on lactate and pyruvate accumulation in the absence of oleate. In contrast, PMA caused a significant increase in lactate and pyruvate accumulation in the absence of oleate, also. The lactate/pyruvate ratio was significantly ($P < 0.05$) increased (more reduced) by A23187 (control; 2.7 ± 0.1, A23187; 4.3 ± 0.3, N = 5), whereas neither PMA nor vasopressin affected it (data not shown). Endogenous ketogenesis (i.e., in the absence of exogenous oleate) was not affected by vasopressin or PMA. However, A23187 produced a marked stimulation of endogenous ketogenesis. The combination of PMA and A23187 did not modify the metabolic effects induced by PMA or A23187 alone.

Dose response of PMA on ketogenesis and glycolysis in the presence of oleate. Increasing concentrations of PMA caused a progressive inhibition of both ketogenesis from 0.5 mM oleate and the formation of acid-soluble products from 0.5 mM [1-^{14}C]oleate (Fig. 1). The maximal effect was seen at 10^{-6} M and was equivalent to 26% inhibition for ketogenesis and 21% inhibition for the formation of acid-soluble products. In accordance with these effects, PMA produced a marked stimulation of lactate and pyruvate accumulation. The maximal effect was seen at 3×10^{-7} M and was equivalent to a 65% increase. In contrast with PMA, the inactive phorbol ester 4α-phorbol-12,13-didecanoate (4α-PDD; 10^{-5} M) did not affect these metabolic processes (Fig. 1).

Effects of vasopressin, PMA and A23187 on the oxidation and esterification of different concentrations of [1-^{14}C]oleate in isolated hepatocytes. The hormonal response of hepatocytes is known to be modified according to the concentrations of added oleate (30). Thus we decided to investigate the effects of agents on the metabolism of various concentrations of [1-^{14}C]oleate, i.e., 0.25 mM, 0.5 mM and 1 mM. The results are summarized in Table 3. Vasopressin (10^{-7} M) inhibited the formation of acid-soluble products from [1-^{14}C]oleate at all three oleate concentrations. Furthermore, the lower the concentration of [1-^{14}C]oleate, the stronger the inhibition, on the basis of percent of control. Vasopressin caused a significant stimulation of $^{14}CO_2$ production from 0.5 mM and 1 mM [1-^{14}C]oleate. The most striking stimulation was observed at 1 mM [1-^{14}C]oleate, whereas vasopressin did not affect $^{14}CO_2$ production at 0.25 mM [1-^{14}C]oleate. Oleate esterification was significantly increased by vasopressin. This action of vasopressin became more marked the lower the oleate concentration. PMA (10^{-6} M) mimicked the inhibitory effect of vasopressin on the formation of acid-soluble products from [1-^{14}C]oleate. The inhibition by PMA also became more marked the lower the concentration of oleate. The esterification of 0.25 mM and 0.5 mM [1-^{14}C]oleate was increased by PMA. The stimulatory effect of PMA on [1-^{14}C]oleate esterification was attenuated with increased concentration of oleate. No significant stimulation of esterification was observed at 1 mM [1-^{14}C]oleate. PMA modestly increased $^{14}CO_2$ production from 1 mM [1-^{14}C]oleate, while it slightly decreased that from 0.25 mM [1-^{14}C]oleate. A23187 (10^{-5} M) exerted only a small effect on [1-^{14}C]oleate metabolism, i.e., a slight decrease in the formation of acid-soluble products from 0.25 mM and 0.5 mM [1-^{14}C]oleate and a slight increase in $^{14}CO_2$ formation from 1 mM [1-^{14}C]oleate.

Metabolic effects of PMA and vasopressin in calcium-depleted hepatocytes. The metabolic actions of vasopressin—i.e., increase in glucose release, stimulation of lactate and pyruvate accumulation, inhibition of ketogenesis, decrease in the formation of acid-soluble products from [1-^{14}C]oleate (0.25 mM) and the stimulation of [1-^{14}C]oleate (0.25 mM) esterification (Tables 1 and 3)—were abolished when hepatocytes were incubated in Krebs-Henseleit buffer containing 1 mM EGTA but no calcium (Table 4). We observed that the effects of vasopressin on 0.5 mM [1-^{14}C]oleate metabolism (Table 3) were also abolished by calcium depletion (data not shown). In contrast with vasopressin, PMA elicited the metabolic actions even in the absence of Ca^{++}, although

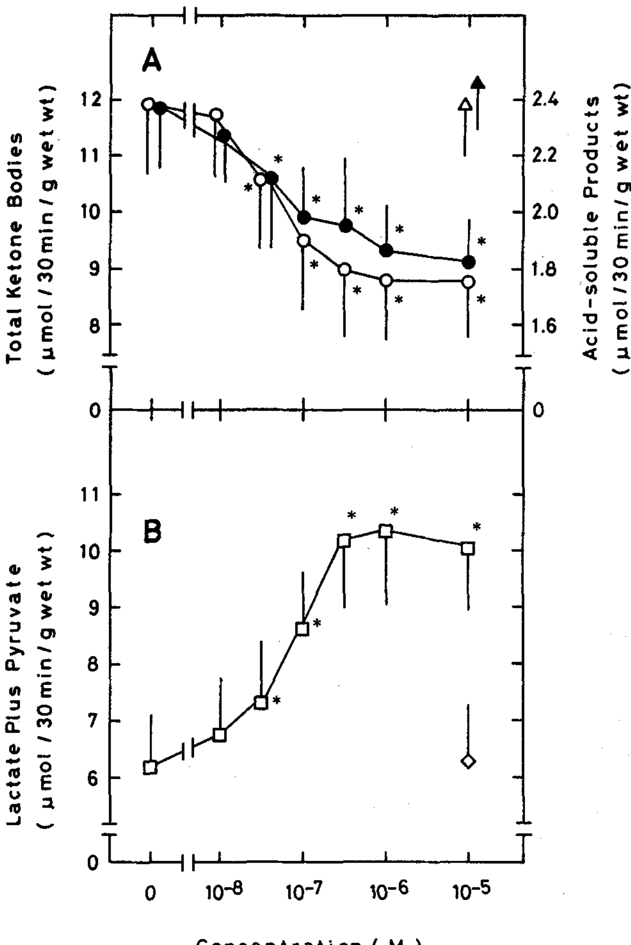

FIG. 1. Effects of phorbol esters on total ketone bodies, acid-soluble products and lactate plus pyruvate. Oleate was 0.5 mM. [1-^{14}C]Oleate was added for the measurement of acid-soluble radioactive products. Incubations were conducted for 30 min. A: effects of 4β-phorbol-12-myristate-13-acetate (PMA, ○) and 4α-phorbol-12,13-didecanoate (4α-PDD, △) on total ketone bodies; effects of PMA (●) and 4α-PDD (▲) on acid-soluble products. B: effects of PMA (□) and 4α-PDD (◇) on lactate plus pyruvate. Each point represents the mean ± SEM of 3-4 hepatocyte preparations. *, $P < 0.05$, compared with control incubations.

the effects on ketogenesis and on the formation of acid-soluble products were somewhat attenuated (Table 4). Stimulation of glucose release by A23187 was completely eliminated by Ca^{++} depletion, whereas that of lactate and pyruvate accumulation was partially eliminated (Table 4).

DISCUSSION

The underlying mechanism of the anti-ketogenic action of vasopressin is not yet understood. Sugden et al. (18) documented that the inhibition of ketogenesis by

TABLE 3

Effects of 4β-Phorbol-12-myristate-13-acetate (PMA), A23187 and Vasopressin on the Oxidation and Esterification of Different Concentrations of [1-^{14}C]Oleate in Isolated Hepatocytes[a]

Metabolic parameter	Oleate (mM)	Control	PMA (10^{-6} M)	A23187 (10^{-5} M)	PMA (10^{-6} M) + A23187 (10^{-5} M)	Vasopressin (10^{-7} M)
Acid-soluble products	0.25	0.67 ± 0.09	0.49 ± 0.07* (73)	0.58 ± 0.08* (87)	0.43 ± 0.07* (64)	0.43 ± 0.07* (64)
	0.5	2.05 ± 0.32	1.55 ± 0.20* (76)	1.78 ± 0.24* (87)	1.42 ± 0.16* (69)	1.44 ± 0.12* (70)
	1	4.51 ± 0.37	3.96 ± 0.31* (88)	4.26 ± 0.36 (94)	3.64 ± 0.31* (81)	3.69 ± 0.23* (82)
$^{14}CO_2$ production	0.25	0.45 ± 0.05	0.40 ± 0.06* (89)	0.49 ± 0.07 (109)	0.43 ± 0.07 (96)	0.51 ± 0.08 (113)
	0.5	0.77 ± 0.10	0.81 ± 0.12 (105)	0.85 ± 0.14 (110)	0.87 ± 0.15 (113)	1.00 ± 0.15* (130)
	1	0.82 ± 0.08	0.94 ± 0.09* (115)	0.92 ± 0.10* (112)	1.02 ± 0.09* (124)	1.24 ± 0.14* (151)
Esterification	0.25	0.54 ± 0.07	0.71 ± 0.08* (131)	0.61 ± 0.08 (113)	0.71 ± 0.08* (131)	0.71 ± 0.07* (131)
	0.5	1.37 ± 0.19	1.69 ± 0.19* (123)	1.48 ± 0.16 (108)	1.69 ± 0.17* (123)	1.73 ± 0.20* (126)
	1	2.73 ± 0.14	2.91 ± 0.22 (107)	2.97 ± 0.20 (109)	3.04 ± 0.30 (111)	3.14 ± 0.19* (115)

[a]Incubations were conducted for 30 min. Results are expressed as μmol oleate utilized/30 min/g wet wt (the mean ± SEM of four hepatocyte preparations). Values in parentheses are percent of control. *, $P < 0.05$, compared with control incubations.

TABLE 4

Effects of 4β-Phorbol-12-myristate-13-acetate (PMA), A23187 and Vasopressin on Oleate Metabolism in the Absence of Calcium[a]

Metabolic parameter	Control	PMA (10^{-6} M)	A23187 (10^{-5} M)	Vasopressin (10^{-7} M)
Glucose release[b]	32.8 ± 4.1	—[d]	31.7 ± 3.9 (97)	30.5 ± 3.9 (93)
Lactate plus pyruvate[b]	4.8 ± 1.4	8.4 ± 2.1* (175)	8.4 ± 1.8* (175)	4.7 ± 1.3 (98)
Acetoacetate plus β-hydroxybutyrate[b]	8.4 ± 0.6	7.1 ± 0.4* (85)	—[d]	8.0 ± 0.6 (95)
Acid-soluble products[c]	0.68 ± 0.09	0.56 ± 0.09* (82)	—[d]	0.64 ± 0.08 (94)
Esterification[c]	0.48 ± 0.06	0.68 ± 0.07* (142)	—[d]	0.49 ± 0.04 (102)

[a]Calcium-depleted hepatocytes were prepared as described in Materials and Methods. They were incubated for 30 min in Krebs-Henseleit buffer containing 1 mM EGTA but no calcium. Results are expressed as the mean ± SEM for four or five hepatocyte preparations from fed rats. Values in parentheses are percent of control. *, $P < 0.05$, compared with control incubations.

[b]Oleate was 0.5 mM. Results are expressed as μmol/30 min/g wet wt.

[c]Since the effects of vasopressin and PMA on these parameters in the presence of calcium were found to be most marked at 0.25 mM [1-^{14}C]oleate (Table 3), the effect of calcium depletion was studied under this concentration. Results are expressed as μmol oleate utilized/30 min/g wet wt.

[d]Since the agents produced less marked or no effects in the presence of calcium (Tables 1 and 3), effects of calcium depletion were not studied.

vasopressin is not a consequence of an increased rate of esterification but is related to increased oxidation of oleate to CO_2. Their conclusion was based on the observation that the increase in esterification of [1-^{14}C]oleate was not dependent on the presence of Ca^{++}, whereas the other effects of vasopressin were. Here, however, we emphasize the involvement of the enhancement of oleate esterification as well as the stimulation of CO_2 production from oleate in the anti-ketogenic action of vasopressin for the following lines of reasoning: First, the suppression by vasopressin of the formation of acid-soluble products from 0.25 mM [1-^{14}C]oleate occurred without any change in $^{14}CO_2$ production but with the stimulation of esterification (Table 3). Second, the data given in Table 3 indicate that the decreased rate of production of acid-soluble products from [1-^{14}C]oleate (0.5 mM and 1 mM) in response to vasopressin can be explained by the sum of the increased rates of $^{14}CO_2$ formation and [1-^{14}C]oleate esterification. Third, it is clear in the present study that the effects of vasopressin on [1-^{14}C]oleate metabolism are all dependent on the presence of Ca^{++} (Table 4). Our previous results (17) demonstrated that vasopressin failed to affect the esterification of 1 mM [1-^{14}C]oleate and with our present results indicate that the role of [1-^{14}C]oleate esterification in the anti-ketogenic action of vasopressin gradually tends to diminish, whereas that of oxidation of [1-^{14}C]oleate to $^{14}CO_2$ increases as the concentration of [1-^{14}C]oleate increases. It has been reported that increased $^{14}CO_2$ production is caused as a consequence of decreased β-oxidation of [1-^{14}C]oleate (31,32). Therefore, increased $^{14}CO_2$ formation from 0.5 mM and 1 mM [1-^{14}C]oleate in response to vasopressin may be not the cause but the result of inhibition of ketogenesis by this hormone.

Our results clearly show that PMA mimicks the effects of vasopressin on oleate metabolism, i.e., inhibition of ketogenesis from oleate, suppression of the formation of acid-soluble products from [1-^{14}C]oleate and stimulation of [1-^{14}C]oleate esterification. We found that another active tumor promotor, 4β-phorbol-12,13-didecanoate (4β-PDD; 10^{-6} M), also significantly inhibited ($P < 0.05$) the formation of acid-soluble products (control; 1.67 $\pm$ 0.21 μmol/30 min/g wet wt, 4β-PDD; 1.30 $\pm$ 0.15 μmol/30 min/g wet wt, n = 3) and stimulated ($P < 0.05$) esterification (control; 1.24 $\pm$ 0.32 μmol/30 min/g wet wt, 4β-PDD; 1.61 $\pm$ 0.21 μmol/30 min/g wet wt, n = 3), when 0.5 mM [1-^{14}C]oleate was added as substrate. The inactive phorbol ester 4α-PDD failed to affect ketogenesis from oleate (Fig. 1). These findings, therefore, strongly suggest that protein kinase C plays an important role in vasopressin actions. Vaartjes and de Haas (33) reported a similar inhibition of ketogenesis from oleate and stimulation of oleate esterification in response to PMA. Kojima et al. (34), on the other hand, documented that PMA alone did not affect the formation of acid-soluble products from [U-^{14}C]palmitate. At this time, we can offer no explanations for the discrepancy between our results and those of Kojima et al. (34). Table 3 shows that the inhibition of the formation of acid-soluble products from 0.25 mM [1-^{14}C]oleate in response to PMA can be attributed to the stimulation of [1-^{14}C]oleate esterification. However, at high concentrations (0.5 mM and 1 mM) of [1-^{14}C]oleate, the contribution of oleate esterification and CO_2 production to the anti-ketogenic action of PMA is less marked than that of vasopressin. We observed that PMA caused a significant inhibition of $^{14}CO_2$ formation from 0.25 mM [1-^{14}C]oleate (Table 3). Vaartjes and de Haas (33) observed similar inhibition with 0.5 mM [1-^{14}C]oleate. We suspect that the decreased CO_2 production as well as suppressed formation of acid-soluble products may be balanced by the accelerated oleate esterification at low concentration of oleate.

In contrast with PMA, A23187 had little effect either on ketogenesis from oleate (Table 1) or on [1-^{14}C]oleate metabolism (Table 3). This suggests that intracellular calcium mobilization is not likely to affect oleate metabolism. Acceleration of endogenous ketogenesis by A23187 (Table 2) was abolished by calcium depletion (data not shown). Calcium mobilization may therefore be involved in the mechanism. Further elucidation of the substrate for the ketone bodies is required.

Neither A23187 nor PMA mimicked the marked stimulatory effect of vasopressin on $^{14}CO_2$ production (Table 3). It remains to be seen whether or not other mediator(s), such as cyclooxygenase product(s), are involved in the regulation.

All three agents stimulated lactate and pyruvate accumulation in the presence of oleate (Table 1). Lactate and pyruvate are known to have an anti-ketogenic action (35). Nevertheless, A23187 failed to affect ketogenesis from oleate. Our data therefore suggest that the stimulated accumulation of lactate and pyruvate does not necessarily lead to a suppression of ketogenesis. Almås et al. (36) reported that the anti-ketogenic action of vasopressin was not the result of increased lactate production. Recently, it has been reported that PMA (33,37) and vasopressin (17,38,39) stimulate fatty acid synthesis but A23187 does not affect it (39). Part of the inhibition of ketogenesis by PMA and vasopressin may be related to the increased fatty acid synthesis, i.e., the elevation of malonyl-CoA levels (40).

We stress that the ability of PMA to affect oleate metabolism was observed even in the absence of extracellular calcium (Table 4). This suggests that PMA stimulates protein kinase C, which has a low requirement for Ca^{++} (15). In contrast to PMA, the effects of vasopressin on oleate metabolism were eliminated by calcium depletion (Table 4). However, if the effects of vasopressin on [1-^{14}C]oleate metabolism (except for $^{14}CO_2$ production) were mediated through the activation of protein kinase C (discussed above), why were these vasopressin actions all abolished by calcium depletion? Bocckino et al. (9) documented that Ca^{++} depletion decreases the ability of vasopressin to increase diacylglycerol. Therefore, Ca^{++} probably mediates some effects of vasopressin on diacylglycerol accumulation. It is also suggested that the involvement of a Ca^{++}-activated kinase(s) is required to elicit vasopressin actions in oleate metabolism.

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FAB MS/MS for Phosphatidylinositol, -glycerol, -ethanolamine and Other Complex Phospholipids

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Fast atom bombardment (FAB) of phosphatidylinositol, phosphatidylethanolamine, cardiolipin, phosphatidic acid and phosphatidylglycerol produces a limited number of very infomative negative ions. Especially significant is the formation of $(M-H)^-$ ions and ions that correspond to the carboxylate portions of these molecules. FAB desorption in combination with collisional activation allows for characterization of fragmentation and determination of structural features. Collisional activation of the carboxylate anion from complex lipids is especially informative. Structural characterization of the fatty acids can be achieved as the released saturated carboxylate anions undergo highly specific charge remote fragmentations that are entirely consistent with the chemistry of carboxylate anions desorbed from free fatty acids. This permits both identification of the modification and assignment of its location on the acid chain. FAB-desorbed alkyl acetyl glycerophosphocholines (platelet-activating factor) do not produce $(M-H)^-$ ions. However, significant high mass ions are formed, and these can be collisionally activated for structural characterization.
Lipids 22, 480–489 (1987).

A variety of mass spectrometry methods has proven useful to analyze phospholipid compounds (1). One method recently shown to be quite useful is fast atom bombardment (FAB) in combination with tandem mass spectrometry (MS/MS) (1–5). We first reported the use of FAB and MS/MS for analysis of phospatidylcholine (2,3) and in subsequent work explored its use for phosphatidylserine (1) and phosphatidylinositol (4). The present work continues the study of phosphatidylinositol and extends these studies to other complex lipids.

Since its development in the early 1980s by Barber et al. (6), FAB has proven to be a very useful desorption method for many labile biomolecules. A number of workers have used FAB for desorbing phospholipid cations (7–16). It was found that $(M+H)^+$ ions of substantial abundance are formed by this method. Although the positive ion mode is used more commonly, FAB mass spectra of negative phospholipid ions also have been studied (1–5,11,17). Phosphatidylcholine typically desorbs to give three high mass anions formed by losses of various portions of the choline moiety and two low mass ions that are the free fatty acid carboxylates from the original complex lipid (1–4). Phosphatidylserine (1,4) and phosphatidylinositol (4,5,17) yield abundant $(M-H)^-$ ions and carboxylate ions corresponding to the two fatty acid components.

Although MS/MS has proven successful for both structural characterization and mixture analysis of a wide range of compounds, reports of its use for phospholipid analysis have been limited (1–5,18). Batrakov et al. (18) used MS/MS methods with electron ionization (EI) to study phospholipids. Typically, molecular ions are either stable or not formed under EI conditions; however, certain high mass ions that are suitable for mixture characterization are produced (18). In addition to our work with FAB MS/MS for analysis of phosphatidylcholines and phosphatidylserine (1–3), both our group (4) and Sherman et al. (5) reported the application of such methods to the study of phosphatidylinositol. The relatively abundant $(M-H)^-$ ions generated by FAB desorption of phosphatidylserine and phosphatidylinositol are well suited to this analysis method. Sherman et al. (5) demonstrated that the fatty acid composition of isomeric phosphatidylinositols can be determined by analyzing the daughter ion spectra even if the constituent fatty acids are similar (e.g., two constituents could be either 18:1 and 18:1, or 18:0 and 18:2). The determination can be made if MS-II has approximately unit resolution, as was demonstrated by using a BEB triple sector MS/MS instrument.

MS/MS methods offer obvious advantages in mixture analysis or for samples of questionable purity. Analysis of spectra of negative daughter ions provides information that is complementary to positive ion analysis. Additional advantages pertain to negative ions because (i) they usually desorb to yield simpler spectra than do positive ions; (ii) they more readily yield ions in the molecular ion region [e.g., $(M-H)^-$ for some compounds, such as phosphatidylserine]; and (iii) they produce abundant carboxylate fragments from complex lipids having esterified acid substituents. Selection and collisional activation of the carboxylate anions is particularly informative. We showed that the identity of the acid, the presence of structural modifications on the acid chain and the identity and location of such modifications may be readily determined from the collisionally activated decomposition (CAD) spectra of carboxylate anions (2,3,19–23).

In this work, we extend previous investigations of phosphatidylinositol and investigate the application of negative ion FAB MS/MS methods to the study of phosphatidylethanolamine, phosphatidylglycerol, cardiolipin, phosphatidic acid and platelet-activating factor (PAF) and its analogs. PAF is an especially interesting phospholipid because it is biologically active.

EXPERIMENTAL PROCEDURES

The Kratos MS-50 triple sector mass spectrometer used in this study is described elsewhere (24). This instrument is equipped with a standard Kratos FAB source and an Ion Tech gun and was operated as described previously for negative ion FAB and CAD analysis (4).

Materials. 1-*O*-(9-Octadecenyl)-2-*O*-acetyl-*sn*-glycero-3-phosphocholine was obtained from Bachem (Bubendorf, Switzerland). Other phospholipids were from Sigma Chemical Co. (St. Louis, Missouri). 1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (PAF), dipalmitoyl *N,N*-dimethyl-phosphatidylethanolamine, dipalmitoyl phosphatidylethanolamine, dipalmitoyl phosphatidic acid, distearoyl phosphatidic acid and dipalmitoyl phos-

phatidylglycerol were obtained as characterized compounds. Fatty acid compositions of phosphatidylinositol from soybean and cardiolipin from bovine heart were not specified by the commercial source.

Triethanolamine, dithiothreitol and dithioerythreitol were obtained from Aldrich (Milwaukee, Wisconsin). Triethanolamine was used as a matrix as supplied. Dithiothreitol and dithioerythritol were mixed in a 5:1 ratio to form the DT matrix.

RESULTS AND DISCUSSION

Negative ions were chosen for this study for several reasons. First, collisionally activated fatty acid fragments produced from complex lipids should yield the same highly distinctive structural information as do the free fatty acid carboxylates desorbed by using FAB. Second, in the positive ion mode, relatively abundant $(M-H)^+$ ions are generated along with $(M+H)^+$ ions of fatty acids, and the former will overlap with other fatty acid fragments containing one or more sites of unsaturation (11). Third, less fragmentation is often observed for negative ions, and hence the process of selecting ions for collisional activation is facilitated.

MS/MS offers the opportunity to study mixtures of both fatty acids and more complex lipids, which is especially useful for samples of questionable purity.

Phosphatidylinositol. The spectra of typical negative phosphatidylinositol ions (**A**) desorbed by FAB have abundant $(M-H)^-$ anions and ions corresponding to the constituent fatty acid carboxylates in the original complex lipid.

A

As the spectrum in Figure 1A shows, phosphatidylinositol from soybeans is a mixture. The principal components are those phosphatidylinositols that give $(M-H)^-$ ions of m/z 833 and 857. However, species that give $(M-H)^-$ ions of m/z 831, 859 and 861 are also present.

Structural characterization of these mixture components as phosphatidylinositols is achieved by selecting and collisionally activating each ion. The principal daughter ions formed from the $(M-H)^-$ ion are carboxylates from the acyl chains, the m/z 241 ion (inositol phosphate–H_2O), the m/z 299 ion formed by loss of both acyl chains (structure **B**), the m/z 297 ion (formally the ion m/z 299-2H), ions formed by the loss of R_1CH_2COOH or R_2CH_2COOH and ions formed by the loss of R_1CHCO and R_2CHCO. The formulas of the ions of m/z 299 and 297 were established as $C_9H_{16}O_9P$ and $C_9H_{14}O_9P$, respectively, by high resolution peak matching.

B

The spectrum of the $(M-H)^-$ ion m/z 833 shown in Figure 1B is representative. Daughter ions of m/z 255 and 279 (labeled b and c in the figure) are carboxylate anions of the 16:0 and 18:2 acid chains, respectively; the m/z 553 ion (e) results from the loss of the 18:2 acid, and the m/z 577 ion (the principal component of peak f) results from the loss of the 16:0 acid. The peak labeled f in the CAD spectrum is broadened considerably, indicating that it may represent multiple ions. The ion m/z 571 is present in the FAB spectrum and hence may be a lesser component of the peak f. This ion is formed by the loss of $C_{16}H_{29}CHCO$ from the $(M-H)^-$ ion. The ion of m/z 595 is the result of the loss of $C_{14}H_{29}CHCO$ from the $(M-H)^-$ ion. Daughter ions of m/z 241 (a) and of m/z 299/297 (d), as noted above, are formed for all phosphatidylinositols and must arise from the glycerol inositolphosphate portion of the molecule. Unfortunately, the resolution of MS-II is not sufficient to resolve doublets spaced by 2 μ. However, the broadened nature of the peaks coupled with the fact that both the m/z 297 and 299 ions are found in the FAB mass spectrum support the existence of a doublet.

Selection by using MS-I and collisional activation of the ions formed by ketene loss and those formed by acid loss from the $(M-H)^-$ ion reveal an important difference in fragmentation behavior. The ions formed by ketene loss fragment to yield a significantly abundant ion of m/z 315 (C_3H_5O + inositol phosphate). The ions formed by loss of RCH_2COOH from the $(M-H)^-$ ion yield no appreciable m/z 315 ion, but rather the ion of m/z 297.

In a similar manner, ions of m/z 831 and 857 in the mixture were characterized as phosphatidylinositols composed of acid chains 16:0, 18:3 and 18:2, 18:2, respectively. Those giving $(M-H)^-$ ions of m/z 859 fragment to produce broadened peaks for the carboxylate daughter ions, which indicates that two species are involved, one with 18:2, 18:1 and the other with 18:3, 18:0 acid chains. Similarly, the $(M-H)^-$ ions of m/z 861 appear to include both 18:2, 18:0 and 18:1, 18:1 species. Clearly, an MS/MS instrument having unit resolution for MS-II would be a significant advantage in studying these phosphatidylinositols.

In addition to the daughter ions described above, a series of high mass daughter ions is present. The fragmentation that produces the ion series appears to be yet another example of the phenomenon we have described as remote-charge-site fragmentation (19–23). Upon collisional activation, closed-shell species that bear long alkyl chains yield a series of fragments arising by losses of the elements of C_nH_{2n+2} from the alkyl terminus remote from the charge site. These charge-remote fragmentations appear to occur via a highly specific 1,4 elimination of H_2, as was previously described (20). The presence of a structural modification

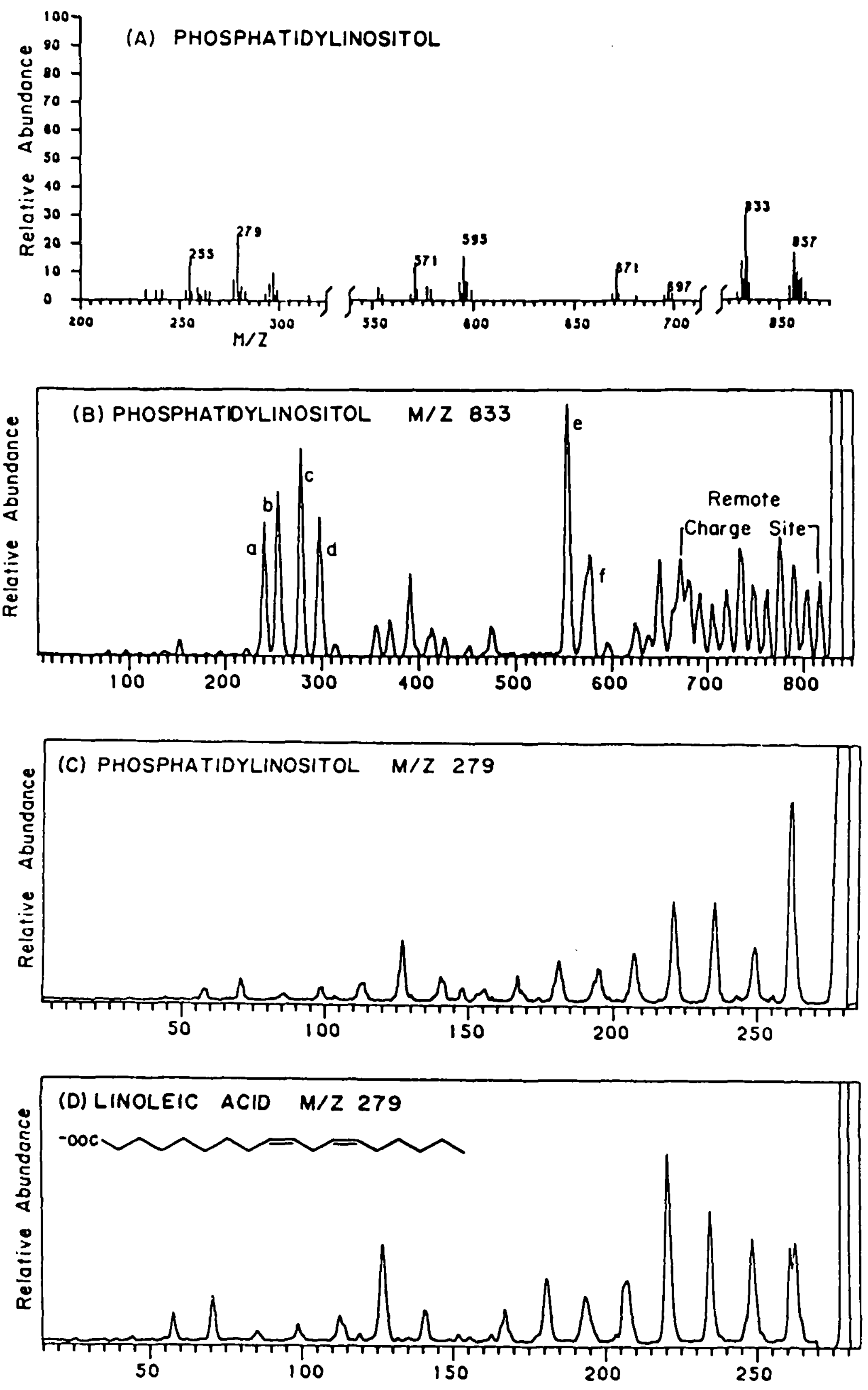


FIG. 1. (A) Mass spectrum of the negative ions produced by fast atom bombardment desorption of a mixture of phosphatidylinositols from soybeans. Ions in the region m/z 830–865 are $(M-H)^-$ ions of the inositols. (B) Spectrum of the daughter ions produced by collisionally activating the $(M-H)^-$ ion, m/z 833. (C) Spectrum of the daughter ions produced by collisionally activating the fragment ion of m/z 279. (D) Spectrum of the daughter ions produced by collisionally activating the $(M-H)^-$ ion, m/z 279, of authentic linoleic acid.

such as branching or a double bond alters the fragmentation pattern. For example, carboxylate anions having a double bond do not transfer a vinylic hydrogen and the double bond is not cleaved. Hence, the fragmentation is interrupted at the site of the double bond.

If only one long alkyl chain is present in the ion of interest, this type of fragmentation provides for highly specific structural characterization of the alkyl chain. Unfortunately, the $(M-H)^-$ anions of phosphatidylinositol contain two long alkyl chains. The remote-charge-site fragmentation of the $(M-H)^-$ ion is of diminished analytical value because it involves the fragmentation of one alkyl chain in competition with the parallel fragmentation of the other.

However, if the carboxylate anion fragments formed as a result of FAB desorption of phosphatidylinositol are selected and collisionally activated, they undergo the expected remote-charge-site fragmentations that allow for specific structural characterization of the acid chain. For example, collisional activation of the ion of m/z 279 (Fig. 1C) and comparison with an authentic sample of linoleic acid $(M-H)^-$ anion of m/z 279 (Fig. 1D) permits identification of the 18:2 acid as linoleic acid. The only notable difference between the two spectra is more abundant water loss for the fragment ion than for the linoleate ion desorbed from the pure acid. The abundant water loss is not unique to this sample, as it is observed typically for collisionally activated linoleate fragment ions. Collisional activation of the ions of m/z 255, 281 and 283 verified their structures as palmitic, oleic and stearic acid carboxylates, respectively. The abundances of the daughter ions formed by water loss are not significantly different than those from the $(M-H)^-$ of the pure acids.

The reason that the water loss is enhanced for the more highly unsaturated fragment ion but not for the others is not understood. However, water loss is a metastable process for carboxylate anions, whereas the remote-charge-site fragmentation is a high energy process seen only under conditions of collisional activation (20). Because the two processes are distinct, perhaps energy differences between the carboxylate desorbed from a free fatty acid and the ion generated as a fragment from a complex lipid are significant.

Characterization of the acid chains of a specific phosphatidylinositol in a mixture by MS/MS is straightforward if isomeric species (e.g., oleic and vaccenic) are not present. Collisional activation of the $(M-H)^-$ anion yields abundant daughter ions that reflect the masses of each of the two carboxylate chains. Carboxylate anions may then be selected for specific characterizations. If, however, two isomeric acid carboxylates are liberated from two different phosphatidylinositols, three stages of tandem mass spectrometry, MS/MS/MS (25), would be necessary to characterize the acid chain for each specific intact lipid.

The FAB MS/MS method offers several advantages over conventional methods in terms of specificity of structural information that may be obtained, especially with regard to the identities of the fatty acids. Because a phosphatidylinositol mixture was analyzed and authentic pure samples were not available, it was not possible to draw conclusions regarding the position of attachment of the acid chains on the phosphatidylinositol. However, on the basis of our previous work with phosphatidylcholine, it is very likely that relative abundances of selected daughter ions of the $(M-H)^-$ ion may indeed reveal such information (1–4).

Although the emphasis of this work is to explore the use of FAB MS/MS methods for qualitative identification and structural characterization of phospholipids, some consideration of quantification was made. For the soybean phosphatidylinositol mixture that was analyzed without prior separation or derivatization, ions corresponding to ca. 95% of the inositol components identified by Myher and Kuksis (26) were observed. Furthermore, the entire FAB MS/MS analysis of the major components of the mixture including collisional activation of the $(M-H)^-$ ions of various inositols was completed in an afternoon. This procedure is much more rapid than conventional methods that involve thin layer chromatographic, enzymatic hydrolysis, separation of hydrolysis products, derivatization of component fatty acids, preparation of trimethylsilyl derivatives of diacylglycerols and then analysis by gas chromatography (26).

Quantification of lipids with FAB MS/MS is not a straightforward process. In previous work with bile salts (27) and fatty acids (23), we found that desorption and/or response may vary considerably between two closely related species [i.e., a 1:1 mixture of two fatty acids may not show $(M-H)^-$ ion abundance in a 1:1 ratio]. However, quantification is possible if an appropriate set of standards is used (27). It is likely that this approach would be necessary for quantification of phosphatidylinositols. Although the relative abundances of the phosphatidylinositol $(M-H)^-$ ions remain relatively constant over the lifetime of the samples, we find that the ion of m/z 833 (the most abundant ion) is ~ 35% of the ions having a mass above m/z 830 (assuming equal response). Myher and Kuksis however report that the component giving this ion is ~ 60% (26).

Thus, although ca. 95% of the expected ions are observed, the distribution of the inositols calculated from the FAB data appears to be different from the reported values (26). This does not negate the value of the FAB MS data, but does underscore the need for caution in quantitative interpretation of the FAB spectrum and the need for use of a series of standards.

Phosphatidylglycerol. Dipalmitoyl phosphatidylglycerol (structure **C**) is representative of phospholipids in which the phosphate is linked with a second unsubstituted glycerol as the head group.

$$
\begin{array}{l}
R_1-CH_2-\overset{O}{\overset{\|}{C}}-O-CH_2 \\
R_2-CH_2-\overset{O}{\overset{\|}{C}}-O-CH \\
\qquad\qquad\qquad\quad H_2C-O-\overset{O}{\overset{\|}{P}}(OH)-O-CH_2-CH(OH)-CH_2OH
\end{array}
$$

C

The negative ion mass spectrum is similar to that of phosphatidylinositol because abundant $(M-H)^-$ ion and ions attributed to the carboxylate fragments are the principal ions observed for both (Fig. 2A). Ions of

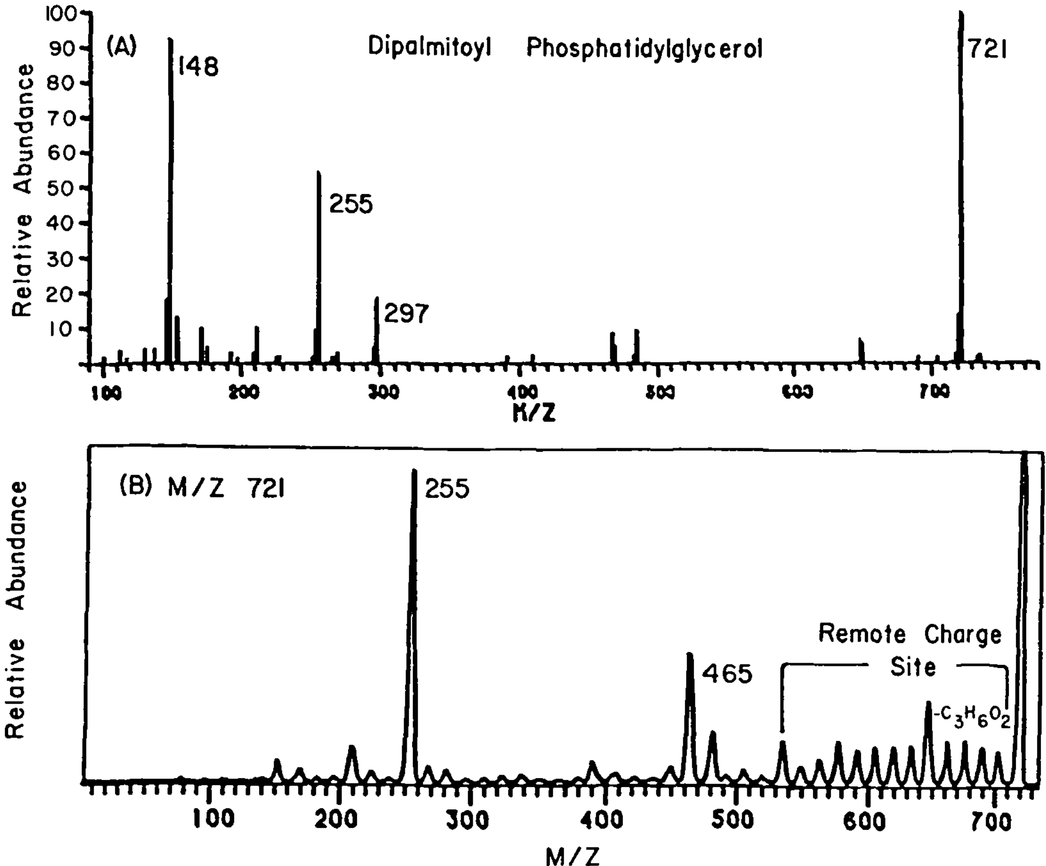


FIG. 2. (A) Mass spectrum of negative ions produced by fast atom bombardment desorption of dipalmitoyl phosphatidylglycerol. (B) Spectrum of daughter ions produced by collisionally activating the $(M-H)^-$ ion, m/z 721.

m/z 148 and m/z 297 are from the triethanolamine matrix.

Collisional activation of the $(M-H)^-$ ion of m/z 721 yields several informative daughter ions (Fig. 2B). The fragment of m/z 647 is formed by loss of $C_3H_6O_2$, presumably involving the loss of the unsubstituted glycerol portion of the molecule with H-transfer to the phosphate oxygen; another of m/z 465 is formed by loss of RCH_2COOH; the ion of m/z 483 is formed by the loss of RCHCO; and another of m/z 255 is attributed to the carboxylate anion. The formulas of the ions of m/z 465 and 483 were established as $C_{22}H_{42}O_8P$ and $C_{22}H_{44}O_9P$ by high resolution peak matching. Remote-charge-site fragmentation is also seen to produce a series of high mass daughter ions formed by losses of C_nH_{2n+2}. Because the constituent acids are the same, the pattern is simple and reflects the saturated nature of each acid. However, as noted for the phosphatidylinositols, remote-charge-site fragmentation of the collisionally activated $(M-H)^-$ anion is of more limited structural value when the acids are different because the fragmentations occurring from the two acyl portions of the molecule are competitive.

The acid chain is best characterized by collisionally activating the carboxylate fragment; for example, the ion of m/z 255 for dipalmitoyl phosphatidylglycerol. The ion undergoes remote-charge-site fragmentation to lose a series of C_nH_{2n+2} fragments and to give a pattern of daughter ions that is characteristic of the palmitic acid $(M-H)^-$ ion.

Cardiolipin. Cardiolipin (structure **D**) is a more complex relative of phosphatidylglycerol.

$R_1-CH_2-C(=O)-O-CH_2$ / $R_2-CH_2-C(=O)-O-CH$ / $H_2C-O-P(=O)(OH)-O-CH_2-CH(OH)-CH_2-O-P(=O)(OH)-O-CH_2$ / $HC-O-C(=O)-CH_2-R_4$ / $H_2C-O-C(=O)-CH_2-R_3$

D

Significant ions in the FAB mass spectrum of one example (Fig. 3A) are those of m/z 1448, 833, 695, 415 and 279. Two points should be noted with regard to these data. First, the highest mass ion observed approaches the mass limit of routine scans on the instrument used. Hence, verification that this was indeed the highest mass ion and that the nominal mass is 1448 u was accomplished using high mass calibration with CsI (the mass of the monoisotopic ion is 1447 u). Second, the spectrum shown is for the second sample of cardiolipin studied. The first sample that we obtained from a commercial source showed these ions as well as sodium adduct ions. The sodium contamination is not a major problem for spectral interpretation because the presence of Na^+ is readily identified by ions spaced by 22 u. However, high sodium ion levels disperse the ionization and reaise the detection limits.

Selection and collisional activation of the principal ions permit further characterization of the compound. The $(M-H)^-$ ion, m/z 1448, fragments to yield ions of m/z 833, 695, 415 and 279, the major ions of the FAB mass spectrum (Fig. 3B). Because the specific composition of the cardiolipin sample was not known, the CAD spectrum provides strong evidence that the sample is indeed a single compound. Formation of the ion of m/z 833 may be attributed to loss of the acyl glycerol portion of one phosphatidic acid group to give daughter ions of structure E.

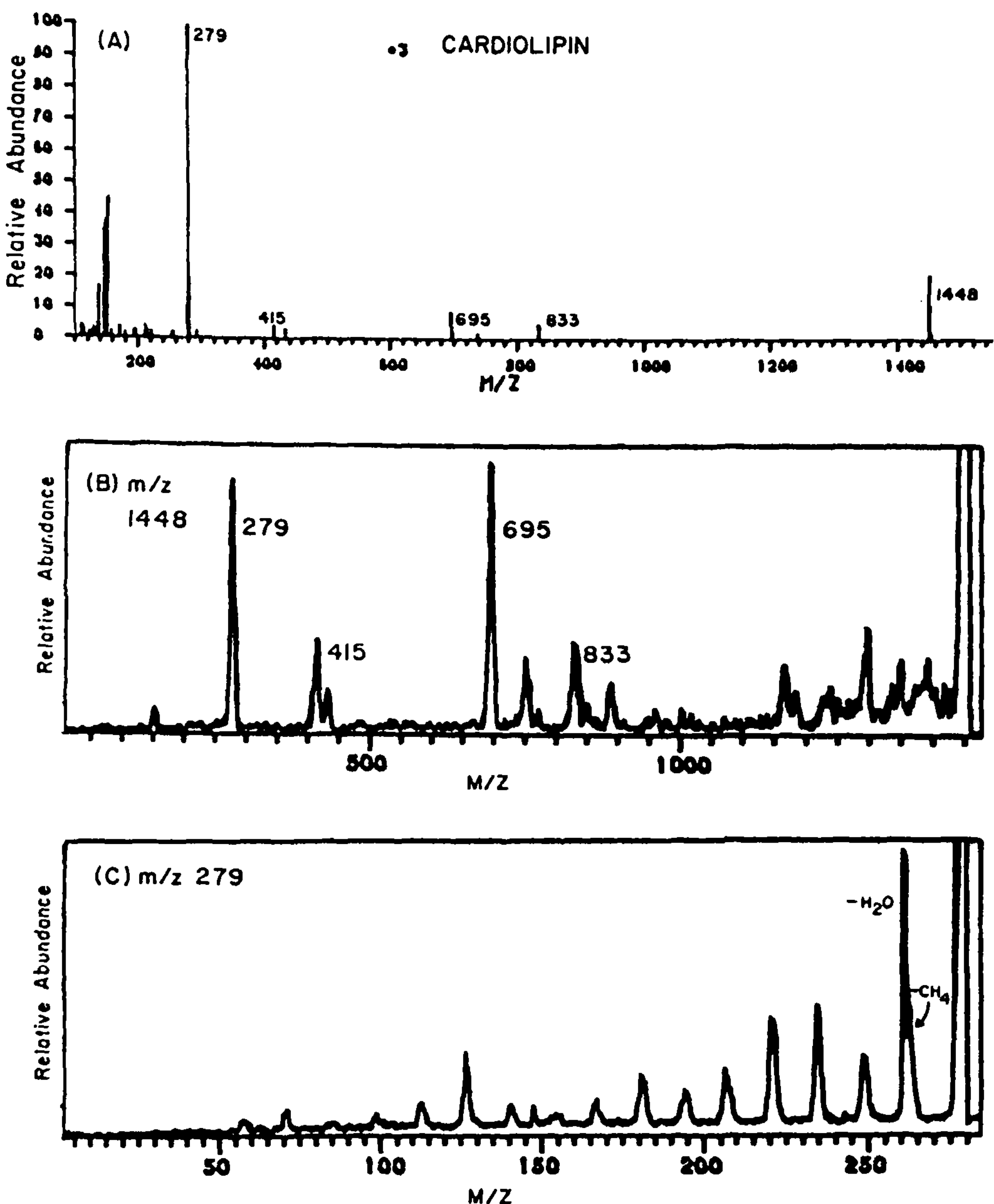


FIG. 3. (A) Mass spectrum of negative ions produced by fast atom bombardment desorption of cardiolipin. (B) Spectrum of the daughter ions produced by collisionally activating the $(M-H)^-$ ion, m/z 1448. (C) Spectrum of the daughter ions produced by collisionally activating the fragment ion of m/z 279.

```
              O
              ‖
R1—CH2—C—O—CH2
              O       |
              ‖       |
R2—CH2—C—O—CH        O
                      |        ‖                                     OH
                    H2C—O—P—O—CH2—CH—CH2—O—P
                               |                  |                  O⁻
                              OH                OH
```

E

Ions of m/z 695 and m/z 415 may be accounted for as a phosphatidic acid ion (structure **F**) and a (phosphatidic acid – RCH_2COOH) ion, respectively.

```
              O
              ‖
R1—CH2—C—O—CH2
              O       |
              ‖       |
R2—CH2—C—O—CH        O
                      |        ‖
                    H2C—O—P—O⁻
                               |
                              OH
```

F

For complex lipids, daughter ions in the mass range of m/z 255 to 311 can usually be attributed to carboxylate anions. The decomposition of the $(M-H)^-$ ion produces only the m/z 279 ion in this mass range. Thus, it is implied that all four acid groups of the cardiolipin are 18:2 acids. Characterization of the ion m/z 279 as the carboxylate anion of linoleic acid is achieved by comparing the collisionally activated decomposition of this ion with that of authentic linoleic acid (compare Figs. 3C and 1D). As for the decomposition product of m/z 279 from the phosphatidylinositol, the water loss is more abundant for the fragment ion than for the fatty acid carboxylate desorbed from the free acid (see above). However, all other fragmentations are comparable.

Collisional activation of the m/z 695 and 415 ions provides further information regarding their structures. The ion of m/z 695, presumably phosphatidic acid, fragments to yield the daughter ions m/z 443 (phospatidic acid – $RCHCO)^-$, m/z 415 (phosphatidic acid – $RCH_2COOH)^-$ and m/z 279 $(RCH_2COO)^-$. The only major daughter ion formed by collisionally activating the ion m/z 415 ion is the ion of m/z 279, which is assigned as the carboxylate anion.

Phosphatidic acid. Phosphatidic acids are the simplest type of phosphoglycerides. They have a glycerol backbone that is esterified with fatty acids at two of the hydroxy groups and a phosphate at the third (see structure **F**). Their fragmentation behavior would be expected to parallel that of the phosphatidic acid portion of the more complex cardiolipin (ion of m/z 695 discussed above). The FAB mass spectra of the distearoyl and dipalmitoyl phosphatidic acids show prominent $(M-H)^-$, $(M-H-RCHCO)^-$ and carboxylate anion daughter ions. These are ions of m/z 647, 409 and 255 for dipalmitoyl phosphatidic acid and ions of m/z 703, 437 and 283 for distearoyl phosphatidic acid.

Collisional activation of the $(M-H)^-$ ions of dipalmitoyl phosphatidic acid causes formation of three daughter ions that correspond to those formed from the phosphatidic acid portion of the cardiolipin molecule (m/z 695 ion). The most abundant daughter ion is the carboxylate anion. The other two daughter ions result from the losses of RCHCO and RCH_2COOH, and the latter loss is slightly favored. For dipalmitoyl phosphatidic acid, these ions are of m/z 255, 409 and 391, respectively. Collisional activation of m/z 409 yields an ion of m/z 153, which arises from the loss of RCH_2COOH from the m/z 409 parent ion, $(M-H-RCHCO)^-$. The acid constituent is determined to be palmitic acid on the basis of collisional activation of the m/z 255 ion, which undergoes the remote-charge-site fragmentation characteristic of a sixteen-carbon saturated acid. Collisional activation of the three distearoyl phosphatidic acid ions yields corresponding results.

Phosphatidylethanolamine. Dipalmitoyl phosphatidylethanolamine and dipalmitoyl *N,N*-dimethyl-phosphatidylethanolamine are examples of phosphoglycerides in which ethanolamine is esterified to the phosphate to form the polar head group (structure **G**).

```
              O
              ‖
R1—CH2—C—O—CH2
              O       |
              ‖       |
R2—CH2—C—O—CH        O
                      |        ‖
                    H2C—O—P—O—CH2—CH2—NH2
                               |
                              OH
```

G

The FAB mass spectrum of dipalmitoyl ethanolamine in triethanolamine matrix is characterized by ions of m/z 732, 716, 690, 647 and 255 (Fig. 4A). The latter three ions may be accounted for as the $(M-H)^-$ ion, $(M-H-C_2H_5N)^-$ ion and the carboxylate anion, respectively. The two higher mass ions appear to be adducts between the ethanolamine head group and the matrix. This assumption is supported by three pieces of evidence. First, these ions are not present if the sample is placed in DT matrix (see Fig. 4A inset). Second, if the two high mass ions are collisionally activated, the daughter ions associated with the carboxylate chains of the ions are identical to those produced by collisional activation of the $(M-H)^-$ ion. Third, the FAB mass spectrum of dipalmitoyl *N,N*-dimethyl phosphatidylethanolamine in triethanolamine matrix is characterized by only ions of m/z 718, 647 and 255; $(M-H)^-$, $(M-H-C_4H_9N)$ and the carboxylate anion, respectively. No high mass adduct ions corresponding to those of m/z 732 and m/z 716 are seen for the *N,N*-dimethyl compound. The absence of adduct ions suggests that matrix molecules are H-bonded via the NH hydrogen atoms. Although the matrix problem with triethanolamine and the unsubstituted ethanolamine does not limit selection and collisional activation of the ions of interest, the adduct ions may be confusing in the analysis of an unknown mixture.

The collisionally activated $(M-H)^-$ ion of m/z 690 of dipalmitoyl phosphatidylethanolamine fragments to yield daughter ions of m/z 452, $(M-H-RCHCO)^-$; m/z 434, $(M-H-RCH_2COOH)^-$; m/z 255, (the carboxylate anion); and a series of high mass daughter ions (see Fig. 4B). The series of high mass daughter ions is that produced by remote-charge-site fragmentation. The high energy requirement for producing remote-charge-site decompositions is again verified by the absence of these ions in the conventional FAB mass spectrum. The ion of

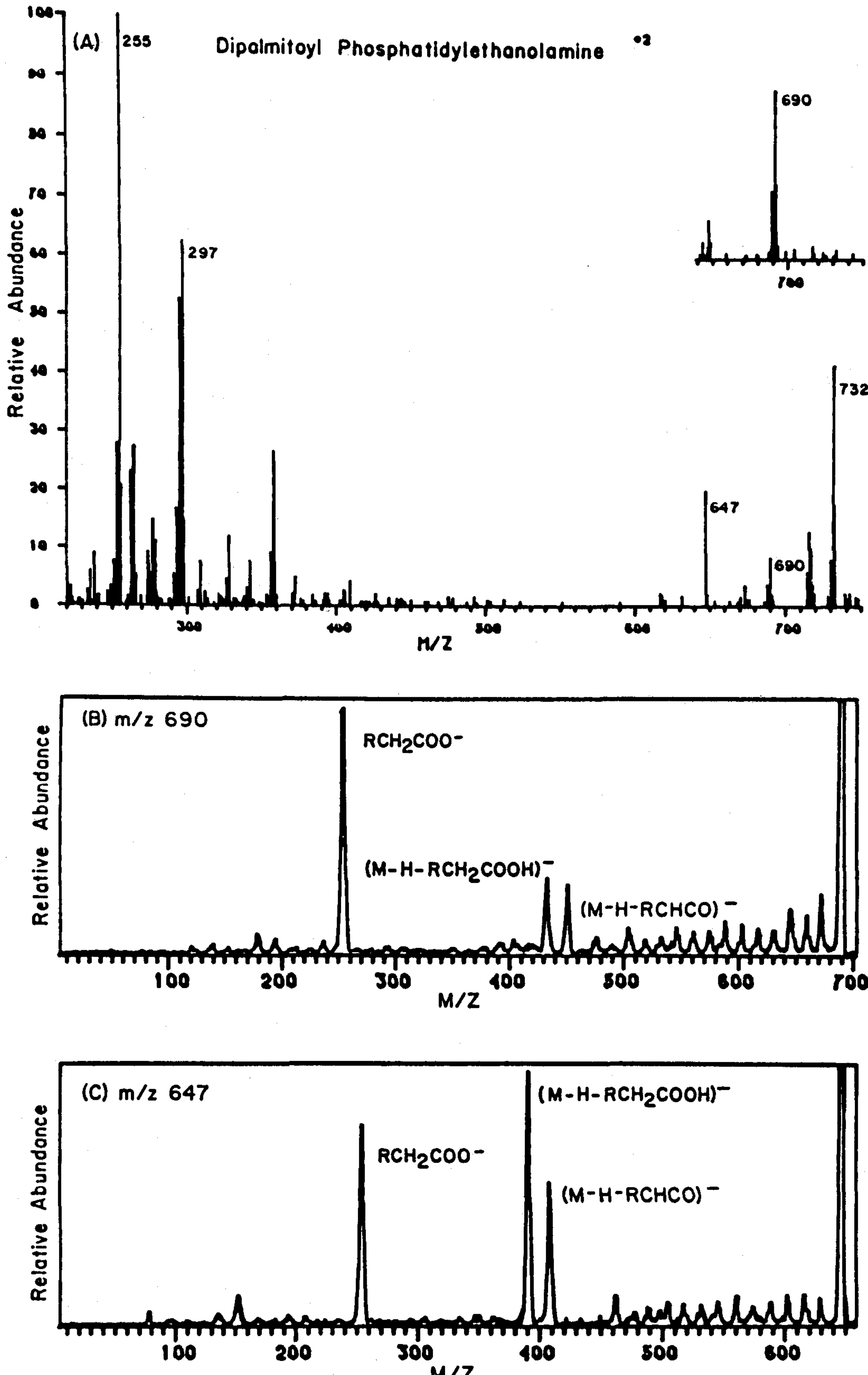
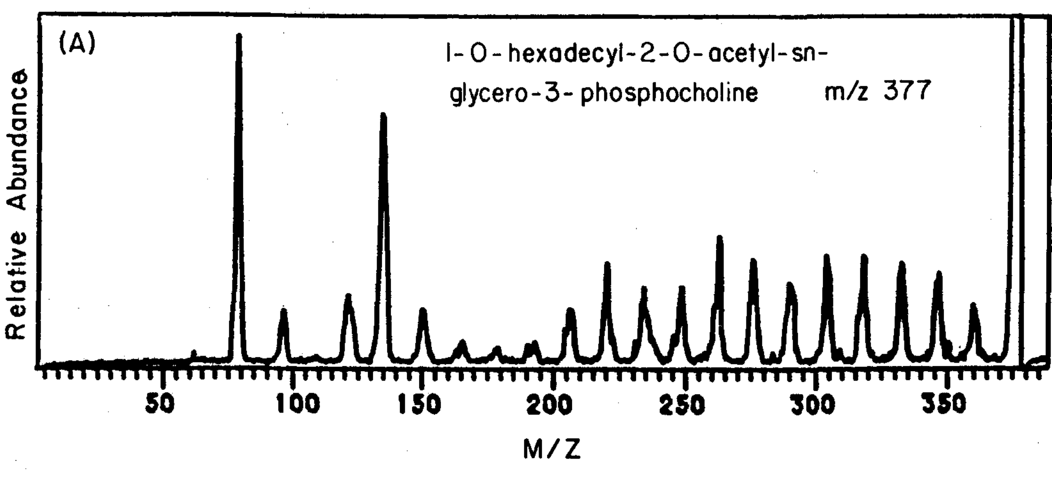


FIG. 4. (A) Mass spectrum of negative ions produced by fast atom bombardment of dipalmitoyl phosphatidylethanolamine. Inset shows spectrum in the mass range m/z 630–750 for sample in DT matrix. (B) Spectrum of the daughter ions produced by collisionally activating the $(M-H)^-$ ion, m/z 690. (C) Spectrum of the daughter ions produced by collisionally activating the fragment ion of m/z 647.



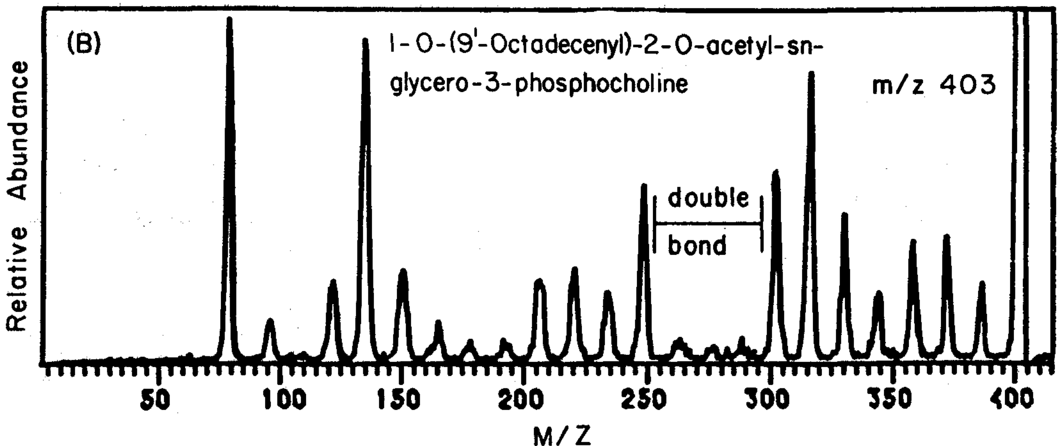


FIG. 5. Spectrum of the daughter ions produced by collisionally activating the fragment ions of (A) m/z 377 of hexadecyl PAF and (B) m/z 403 of the 9-octadecenyl platelet-activating factor analog.

m/z 647 from phosphatidylethanolamine fragments upon collisional activation to yield the same principal daughter ions as does the $(M-H)^-$ ion (Fig. 4C), but the relative abundances of these ions are altered. For the $(M-H)^-$ ion, the formation of the carboxylate anion is favored, but the abundance of the acid carboxylate relative to ions formed by the losses of RCHCO and RCH_2COOH is substantially diminished for the $(M-H-C_4H_9N)^-$ ion. Furthermore, the loss of RCH_2COOH is favored over the loss of RCHCO by a factor of 3:2 for the $(M-H-C_4H_9N)^-$ ion, whereas these ions are produced in nearly equal abundance from the $(M-H)^-$ ion. The ion of m/z 255 is readily characterized as the palmitate anion as it undergoes the typical remote-charge-site fragmentation of the long chain, unbranched, saturated acid. The collisionally activated fragmentations of the principal ions of dipalmitoyl *N,N*dimethyl-phosphatidylethanolamine parallel those of the unsubstituted phosphatidylethanolamine.

Platelet-activating factor and its analogs. 1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (PAF; structure **H**) and 1-*O*-(9'-octadecenyl)-2-*0*-acetyl-*sn*-glycero-3-phosphocholine (structure **I**) differ from the more common phosphatidylcholines because the long aliphatic chain at the one position is attached by an ether rather than an ester linkage. Five major ions are seen in the FAB mass spectra of these compounds. These ions are of m/z 508, 463, 437, 377 and 153 for the hexadecyl PAF and of m/z 534, 489, 463, 403 and 153 for the 9-octadecenyl analog. These ions may be accounted for as $(M-CH_3{}^+)^-$, [M − HN

CH_2O-CH_2 (hexadecyl chain)
$CH-O-C(=O)-CH_3$
$CH_2-O-P(=O)(O^-)-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$

H

CH_2OCH_2 (9-octadecenyl chain)
$CH-O-C(=O)-CH_3$
$CH_2-O-P(=O)(O^-)-O-CH_2-CH_2-\overset{+}{N}(CH_3)_3$

I

$(CH_3)_3{}^+]^-$, $(M-C_5H_{12}N^+)^-$, $[M-(C_5H_{12}N^+ + CH_3COOH)]^-$ and $C_3H_6PO_5^-$. The three highest mass ions are analogous to those previously observed for phosphatidylcholines for which the fatty acid chains are esterified to glycerol (1–3). However, there are no significantly abundant ions that may be directly attributed to only the alkyl ether portion of the molecule. This is in sharp contrast to the fragmentation of the phospholipids for which the long chain is esterified to the glycerol backbone. Cleavage of the ester linkage and formation of the carboxylate anion are often the principal fragmentations of these molecules (1–3).

PAF and its analogs, in a manner similar to phosphatidylethanolamine, form adduct ions with the triethanolamine matrix. The adduct ion, (M + 62), is very abundant. Hence, DT was used instead of triethanolamine as the matrix for the analysis.

Collisional activation of the three highest mass ions (i.e., the ions of m/z 508, 463 and 437) of the hexadecyl PAF yields the same fragmentation. All fragment to give two daughter ions by the loss of the acetyl moiety. One ion is formed by the loss of CH_3CHCO and the other by the loss of CH_3COOH. The 9-octadecenyl high mass ions fragment in the same moderately informative fashion.

However, collisional activation of the $[M-(C_5H_{12}N^+ + CH_3COOH)]^-$ ion, the ions of m/z 377 and 403 for hexadecyl PAF and the 9-octadecenyl PAF analog, respectively, causes remote-charge-site fragmentation, which permits specific characterization of the alkyl chain (Fig. 5). For the saturated hexadecyl ion, the entire series of C_nH_{2n+2} losses for the sixteen-carbon chain (Fig. 5A) is observed, although relative abundances are decreased as the ether linkage is approached. The presence and position of a double bond in the octadecenyl chain are identified by the very low abundance of three daughter ions (see Fig. 5B) (19,20). The position of the double bond in the C_{18} chain is readily assigned to be eight carbons from the alkyl terminus by correlating the daughter ions of the series with the carbon atoms of the alkyl chain starting with the highest mass daughter ion and the terminal carbon of the chain.

In summary, the combination of FAB for producing negative ions and MS/MS for collisionally activating them is useful for determining phospholipids and their fatty acid components. This is demonstrated by the results in this paper as well as those in our previous publications on phospholipids (1–3) and other complex lipids (28). The method is particularly useful for characterizing fatty acid components directly from the complex lipid without a prior degradation or derivatization. As demonstrated for the phosphatidylinositol, the method is applicable to mixtures, and sample purity is not critical.

ACKNOWLEDGMENTS

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Fatty Acid Composition of Seeds from the Australian *Acacia* Species[1]

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Presented are the lipid content and fatty acid composition of 20 species of edible Australian *Acacia* seeds. Aborigines reportedly have used at least 18 of these as foods. Seed lipid content ranged from 3% to 22%, with an average of 11% on a dry weight basis. Linoleic (12–71%), oleic (12–56%) and palmitic (7–35%) acids were the major fatty acids. Smaller proportions of behenic, stearic and vaccenic acids were detected. Seventeen of the 20 species were found to have polyunsaturated to saturated (P/S) fatty acid ratios greater than 1, with four species having ratios in excess of 4. The persistent arils attached to the seeds of certain Australian *Acacias* and containing a portion of the total lipid were associated with a significantly reduced proportion of linoleic acid in the total seed material. This observation was explained by the aril lipid possessing a markedly different fatty acid composition from that of the seed lipid. For comparison, seeds from two non-Australian *Acacia* species (*A. farnesiana* and *A. cavenia*) were analyzed. Australian and non-Australian were found to exhibit markedly different fatty acid profiles.
Lipids 22, 490–494 (1987).

The leguminous plant family Mimosaceae contains over 1,000 *Acacia* species. *Acacia* is a genus of mainly shrubs or small trees found in many parts of the tropics and southern temperate regions (1). *Acacia* species have been put to many uses, notably for dyes, soaps, perfumes, timber, forage and soil conditioning. For instance, the highly scented flowers of *A. farnesiana* are used in the south of France for perfume manufacture (2). The thorns of *A. farnesiana* have been used by some aboriginal peoples to deaden the pain of snakebite (3). The nitrogen-fixing capability of *A. holosericea* has been found useful in land rehabilitation after mining in the Northern Territory of Australia (4), and the foliage and pods of some species (*A. aneura, A. farnesiana, A. victoriae*) are valuable supplements to stock feed (1,2). In addition, milled seeds from *A. coriacea* have been used to supplement wheat flour in the production of a palatable bread (5).

The seeds of some Australian *Acacias* used as food by aborigines have been assessed for gross nutritional composition (6–8) and have been shown to contain significant amounts of lipid. Yet seeds from only four species, not recorded as edible, have been analyzed for fatty acids (9,10). This study reports the fatty acid composition of seeds from 20 Australian *Acacia* species. For comparison, two nonindigenous species were also analyzed.

MATERIALS AND METHODS

Seed collection. Seeds from all but two of the Australian *Acacia* species analyzed were selected on the basis of their reported use as aboriginal foods (11). Seeds from *A. concurrens* and *A. crassicarpa*, not reported as edible, were included to compare with the "edible" species. Two non-Australian *Acacia* species were also analyzed: *A. farnesiana,* collected in India, and *A. cavenia,* a Chilean species grown in the Royal Botanic Gardens, Sydney, Australia. Seeds from the remaining species were collected throughout Australia, mostly in the Northern Territory (Fig. 1). Some seeds were collected with the help of local aborigines as part of the bush food analysis program of the University of Sydney; others were supplied by CSIRO Division of Forest Research (Canberra, Australian Capital Territory) and by M.L. Farrar Pty Ltd (Nowra, New South Wales).

Sample storage and handling. Seeds were stored at ambient temperature for up to 6 weeks before milling in a Fritsch Pulverisette food mill (John Morris Scientific Pty Ltd, Sydney, Australia). The flours were freeze-dried and stored at −15 C.

Lipid extraction. Lipids were extracted from varying amounts (2 to 5 g) of the milled, freeze-dried samples using chloroform/methanol (2:1, v/v) and a wash according to the method of Folch et al. (12,13). After removal of solvent, the lipids were dissolved in petroleum ether (bp 40–60 C, 2 × 10 ml) and transferred to a tared flask, the solvent was evaporated under reduced pressure and the lipids were quantified gravimetrically. The lipids were redissolved in petroleum ether and stored in glass vials under nitrogen at −15 C.

Preparation of fatty acid methyl esters. Replicate samples of the lipid (10 to 100 mg) were saponified, and the fatty acids were methylated using a method based on that of Oulaghan and Wills (14). If not analyzed immediately, the fatty acid methyl esters in CH_2Cl_2 were stored under nitrogen at −15 C.

Gas chromatography. The esters were analyzed by flame ionization gas chromatography (Hewlett Packard HP 5890A capillary gas chromatograph), using a BP10 capillary column (12.5 m × 0.22 mm id) with hydrogen as carrier gas (flow rate 2.8 ml/min; oven temp 180 C; injector temp 250 C; detector temp 300 C).

Mean percentage fatty acid composition was calculated after integration (HP 3392A integrator) by using response factors derived from results with standard mixtures of known composition run on the same column under identical conditions. The standard fatty acid mixtures were obtained from Supelco (Bellefonte, Pennsylvania).

Using a methyl silicone fluid column at an oven temperature of 160 C, vaccenic acid (18:1n-7; $RRT_{18:0} = 0.892$) was resolved from oleic acid (18:1n-9; $RRT_{18:0} = 0.880$) and identified by cochromatographing with standard *cis*-vaccenic acid methyl ester (Sigma, St. Louis, Missouri).

RESULTS AND DISCUSSION

The *Acacia* seeds analyzed contained between 3% (*A. crassicarpa*) and 22% (*A. adsurgens, A. tetragonophylla*) lipid on a dry weight basis (Table 1), with an average lipid content of 11 + 5% (mean + SD, n = 20).

The polyunsaturated to saturated (P/S) fatty acid ratios

[1]Some of this work published as short report in *Proc. Nutr. Soc. Aust. 10*, 209–212 (1985).

TABLE 1

Total Lipid (% Dry Wt), Contribution of Aril Lipid to Total Lipid and % Proportion of Fatty Acids for Seeds from 20 Australian *Acacia* Species[a]

	1	2	3	4	5	6A	6B	7	8	9A	9B	10	11	12	13	14	15	16	17	18	19	20A	20B
Total lipid	8	22	12	19	10	15	10	3	9	12	11	11	11	11	10	4	6	12	14	16	22	4	3
Aril lipid ÷ total lipid	4	30	0	20	39	64	0	99	1	3	0	12	47	30	18	1	19	2	14	62	79	1	2
Fatty acid																							
14:0	—[b]	tr[c]	—	tr	—	—	—	—	—	tr	—	—	—	—	tr	—	tr	—	tr	—	—	—	—
16:0	8	17	9	16	19	20	7	35	7	7	7	8	16	12	15	9	7	10	10	15	17	8	8
18:0	2	4	1	2	4	3	2	2	3	2	2	3	3	4	4	6	4	2	3	3	3	2	3
20:0	2	1	1	1	1	1	1	tr	1	1	1	1	1	1	2	4	1	2	1	1	1	2	2
22:0	7	1	3	5	2	3	7	—	3	5	7	3	1	2	4	5	3	6	4	2	1	4	4
24:0	1	—	1	tr	—	—	1	—	—	1	tr	—	—	—	1	2	1	1	1	—	—	2	1
Σ Saturates (S)	19	24	15	23	27	26	18	37	15	16	16	15	22	20	26	26	18	22	19	22	21	17	18
16:1	tr	2	tr	tr	tr	1	tr	2	—	tr	—	tr	1	1	tr	tr	tr	tr	tr	2	1	tr	tr
18:1	13,1[d]	38,3	17,1	21,1	53,2	39,2	15;1	43,5	14,1	12,1	12,1	14,1	36,3	29,1	24,1	19,1	18,1	34,1	19,1	32,2	56,5	27,1	26,1
20:1	tr	—	1	tr	1	tr	1	tr	—	tr	—	tr	tr	tr	tr	tr	tr	1	tr	—	tr	1	tr
Σ Monoenes	14	43	20	23	57	43	16	50	15	14	14	15	40	31	26[f]	19	20	36	21	37	63	29	28
18:2	66	32	65	53	17	30	64	12	70	71	70	70	36	49	46	54	62	43	59	41	16	52	54
18:3	1	tr	tr	1	tr	1	tr	—	tr	1	tr	tr	1	1	1	tr	1	tr	tr	tr	tr	1	tr
Σ Polyenes (P)	67	33	65	54	17	31	65	12	70	71	71	71	38[g]	49	47	55	62	43	59	41	16	53	54
P/S[e]	3.5	1.4	4.4	2.3	0.6	1.2	3.5	0.3	4.8	4.5	4.4	4.9	1.7	2.5	1.8	2.1	3.6	2.0	3.0	1.9	0.8	3.1	2.9

[a]1, *A. acradenia;* 2, *A. adsurgens;* 3, *A. aneura;* 4, *A. concurrens;* 5, *A. coriacea;* 6A, *A. cowleana* (+ arils); 6B, *A. cowleana* (− arils); 7, *A. crassicarpa;* 8, *A. dictyophleba;* 9A, *A. holosericea* (Alice Springs, Northern Territory); 9B, *A. holosericea* (Mt. Isa, Queensland); 10, *A. kempeana;* 11, *A. ligulata;* 12, *A. longifolia;* 13, *A. lysiphloia;* 14, *A. monticola;* 15, *A. murrayana;* 16, *A. oswaldii;* 17, *A. stipuligera;* 18, *A. tenuissima;* 19, *A. tetragonophylla;* 20A, *A. victoriae* (Utopia, Northern Territory); 20B, *A. victoriae* (Alice Springs, Northern Territory).

[b]—, Not detected.

[c]tr, Trace proportion (less than 0.5%).

[d]% Proportion of oleic acid (n-9), % proportion of vaccenic acid (n-7).

[e]Polyunsaturated to saturated fatty acid ratio.

[f]Includes 1% of a component tentatively identified as an isomer of 20:1.

[g]Includes 1% of a component identified as 16:3 (n-3).

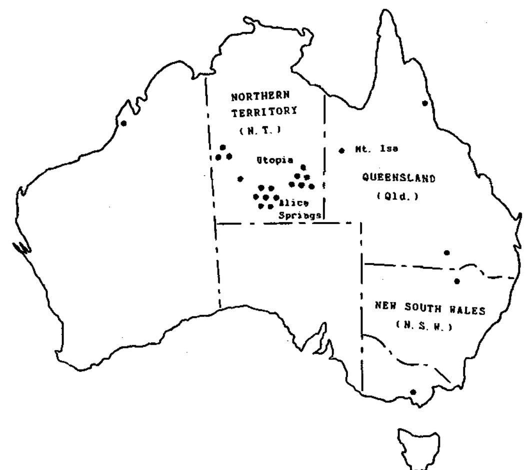

FIG. 1. Collection sites for the Australian *Acacia* seeds; n = 23 (species are listed in Table 2).

ranged from 0.3 to 4.9. Seventeen of the 20 Australian *Acacia* species had P/S ratios greater than 1; four had ratios in excess of 4. The polyunsaturated fatty acids comprised almost exclusively linoleic acid (18:2n-6), with linolenic acid (18:3n-3) accounting for less than 2% of the total fatty acids (Table 1).

In 14 of the *Acacia* seed lipids analyzed, linoleic acid was the major fatty acid (41–71%), with oleic acid and its isomer, vaccenic acid (13–35%), and palmitic acid (7–16%) being most of the remaining fatty acids. Behenic acid (22:0) was also present (2–7%), a phenomenon often associated with Leguminosae seeds (15).

The fatty acid profiles for these 14 *Acacia* seed lipids were in general agreement with those found by other workers. In particular, they were similar to the profiles obtained by Rivett et al. (10) for seeds from three other Australian *Acacias (A. alata, A. dealbata, A. drummondii). A. cyclops*, a native Australian plant introduced to South Africa as an anchorage for the windswept soil of Cape Flats, contained similarly high proportions of linoleic acid (68%) with oleic and palmitic acids being the other major components (10% and 6%, respectively) (10).

The remaining six Australian species (*A. adsurgens, A. coriacea, A. cowleana, A. crassicarpa, A. ligulata, A. tetragonophylla*) analyzed in our study differed from the general trend. They contained significantly lower proportions of linoleic acid (12–36%), significantly higher proportions of oleic acid and its n-7 isomer, vaccenic acid (41–61%), and higher proportions of palmitic acid (16–35%). The slight variations in latitude, altitude and other growing conditions should not cause such differences, since the collection sites were quite similar (Fig. 1). In addition, two samples of *A. holosericea* analyzed from different collection sites (Alice Springs in the Northern Territory and Mt. Isa in Queensland) (Fig. 1) had almost identical fatty acid compositions. A similar situation resulted for the two different samples of *A. victoriae* analyzed (Table 1), indicating that factors such as latitude are unlikely to be responsible for this difference.

Effect of arils on composition. An aril, elaiosome or funicle is the short stalk attaching the seed to the pod; in some *Acacia* species, it remains with the seed. Several *Acacia* species were noted to possess large arils relative to the seed, and in these, aril lipid contributed greatly to the total lipid of the seed material (99% for *A. crassicarpa)* (Table 1). O'Dowd and Gill (16) have measured the lipid content (but not fatty acid composition) of arils and seeds from five of the species (*A. aneura, A. cowleana, A. ligulata, A. longifolia* and *A. tetragonophylla*) reported here. In their results, the percentage of aril lipid in the total seed lipid ranged from 5% (*A. aneura*) to 76% (*A. tetragonophylla*).

TABLE 2

Total Lipid (% Wet Wt) and % Proportion of Fatty Acids for Arils from four Australian *Acacia* Species

	A. adsurgens	*A. coriacea*	*A. cowleana*	*A. tenuissima*
Total lipid	37	31	50	52
Fatty acid				
14:0	—[a]	tr[b]	tr	—
16:0	23	24	35	27
18:0	3	5	2	3
20:0	tr	tr	tr	tr
22:0	tr	—	—	—
Σ Saturates (S)	27	30	37	30
16:1	3	tr	4	4
18:1	62,5[c]	61,2	49,3	57,5
20:1	tr	tr	tr	—
Σ Monoenes	69	64	56	66
18:2	4	4	6	2
18:3	1	1	1	1
Σ Polyenes (P)	4	5	7	3
P/S[d]	0.2	0.2	0.2	0.1

[a]—, Not detected.

[b]tr, Trace proportion (less than 0.5%)

[c]% proportion of oleic acid (n-9), % proportion of vaccenic acid (n-7).

[d]Polyunsaturated to saturated fatty acid ratio.

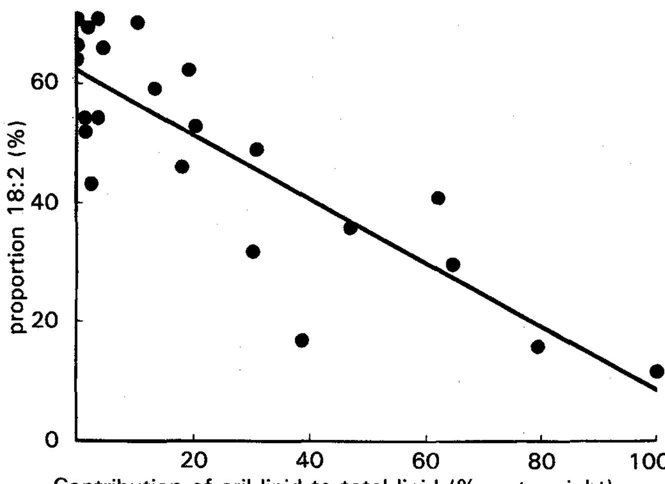

FIG. 2. Linear relationship between the linoleic acid proportion and the extent to which the aril contributes to the total seed lipid for Australian *Acacia* species. n = 23; equation of line: y(%) = 62 − 0.53x, r = −0.840, p < 0.01.

In the aril fatty acids of four *Acacia* species (*A. adsurgens, A. coriacea, A. cowleana* and *A. tenuissima*), oleic acid and its isomer (18:1n-7) constituted over 50% of the total fatty acids; palmitic acid was the other major constituent (23–35%) (Table 2). Linoleic acid was present only in small proportions (2–6%), and there was little or no behenic acid. Black et al. (9) reported similar proportions for the aril lipid composition of *A. cyclops.*

In this study, a significant negative correlation ($r = -0.840$, $p < 0.01$) was found between the linoleic acid proportion and the extent to which aril lipid contributed to total seed lipid (Fig. 2). With an increasing contribution from the arils to the total lipid content of the seed material, there is a decrease in the linoleic acid proportion and a corresponding increase in palmitic and oleic acids. In support of this, *A. cowleana* seeds analyzed without arils were found to be richer in linoleic acid than the arillate sample and correspondingly lower in oleic and palmitic acids (Table 1).

Australian vs non-Australian Acacia species. We analyzed the non-arillate seeds from a Chilean *Acacia* (*A. cavenia*) grown in Sydney, Australia. It contained 54% linoleic acid, which is lower than would be expected for non-arillate Australian species (Fig. 2, intercept 62%). *A. farnesiana* seeds collected from India were also analyzed. Its seeds, like *A. cavenia,* were non-arillate and, like this Chilean species, the Indian sample contained only 54% linoleic acid.

Fatty acid analyses of seeds from seven African *Acacia* species have been reported. Six were from northern Sudan (17), and the seventh was a South African native (18). By comparison, Australian species of *Acacia* generally have higher proportions of linoleic acid and correspondingly lower proportions of oleic and palmitic acids than their African counterparts. We find no mention of persistent arils in the African species analyzed; hence, genetic factors seem a more probable cause for the differences observed. In support of this contention, we note that *A. cyclops* (9), an Australian native introduced to South Africa, exhibits a characteristically "Australian" fatty acid profile.

These data substantiate the suggestion (19) that the Australian *Acacia* may be taxonomically distinct from *Acacias* originating elsewhere in the world.

TABLE 3

Total Lipid (% Dry Wt) and % Proportion of Fatty Acids for Seeds from Two Non-Australian *Acacia* Species

	A. cavenia[a]	*A. farnesiana*[b]
Total lipid	3	3
Fatty acid		
14:0	tr[c]	tr
16:0	14	14
18:0	6	5
20:0	1	2
22:0	tr	3
24:0	—[d]	1
Σ Saturates (S)	21	24
16:1	tr	tr
18:1	24,1[e]	19,1
20:1	tr	tr
Σ Monoenes	25	20
18:2	54	54
18:3	tr	1
Σ Polyenes (P)	54	55
P/S[f]	2.7	2.3

[a]A Chilean native grown in the Royal Botanic Gardens, Sydney, Australia.
[b]Collected from India.
[c]tr, Trace proportion (less than 0.5%).
[d]—, Not detected.
[e]% proportion of oleic acid (n-9), % proportion of vaccenic acid (n-7).
[f]Polyunsaturated to saturated fatty acid ratio.

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Metabolism in Humans of *cis*-12,*trans*-15-Octadecadienoic Acid Relative to Palmitic, Stearic, Oleic and Linoleic Acids

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Mixtures of triglycerides containing deuterium-labeled hexadecanoic acid (16:0), octadecanoic acid (18:0), *cis*-9-octadecenoic acid (9c-18:1), *cis*-9,*cis*-12-octadecadienoic acid (9c, 12c-18:2) and *cis*-12,*trans*-15-octadecadienoic acid (12c,15t-18:2) were fed to two young-adult males. Plasma lipid classes were isolated from samples collected periodically over 48 hr. Incorporation and turnover of the deuterium-labeled fats in plasma lipids were followed by gas chromatography-mass spectrometry (GC-MS) analysis of the methyl ester derivatives. Absorption of the deuterated fats was followed by GC-MS analysis of chylomicron triglycerides isolated by ultracentrifugation.

Results were the following: (i) endogenous fat contributed about 40% of the total fat incorporated into chylomicron triglycerides; (ii) elongation, desaturation and chain-shortened products from the deuterated fats were not detected; (iii) the polyunsaturated isomer 12c,15t-18:2 was metabolically more similar to saturated and 9c-18:1 fatty acids than to 9c,12c-18:2; (iv) relative incorporation of 9c,12c-18:2 into phospholipids did not increase proportionally with an increase of 9c,12c-18:2 in the mixture of deuterated fats fed; (v) absorption of 16:0, 18:0, 9c-18:1, 9c,12c-18:2 and 12c,15t-18:2 were similar; and (vi) data for the 1- and 2-acyl positions of phosphatidylcholine and for cholesteryl ester fractions reflected the known high specificity of phosphatidylcholine acyltransferase and lecithin:cholesteryl acyltransferase for 9c,12c-18:2.

These results illustrate that incorporation of dietary fatty acids into human plasma lipid classes is selectively controlled and that incorporation of dietary 9c,12c-18:2 is limited. These results suggest that nutritional benefits of diets high in 9c,12c-18:2 may be of little value to normal subjects and that the 12c,15t-18:2 isomer in hydrogenated fat is not a nutritional liability at the present dietary level.
***Lipids 22*, 495–504 (1987).**

Hydrogenated vegetable oil, which is used for the production of margarines, cooking oils and shortenings, contains a variety of mono- and polyunsaturated fatty acid isomers. In a recent report prepared for the Food and Drug Administration, per capita consumption in the U.S. of *trans* fatty acids from hydrogenated vegetable oils was estimated at about 8 g/day or ca. 6% of total fat (1). Results summarized in various review articles indicate that previous studies with specific isomeric fatty acids have investigated mainly the metabolism of the *trans* and *cis* positional isomers of octadecenoic acid and *trans,trans*-octadecadienoic acid isomers (1–4). Relatively few studies have investigated the *cis,trans*- and *trans,cis*-octadecadienoic acid isomers. No human nutritional or metabolic data are available for the geometrical isomers of 12,15-octadecadienoic acid, which are formed from linolenic acid during partial catalytic hydrogenation (5).

An objective of this study was to compare in humans the relative absorption and incorporation of *cis*-12,*trans*-15-octadecadienoic acid (12c,15t-18:2) to hexadecanoic acid (16:0), octadecanoic acid (18:0), *cis*-9-octadecenoic acid (9c-18:1) and *cis*-9,*cis*-12-octadecadienoic acid (9c,12c-18:2). The study also provided an opportunity to determine the extent to which deuterium-labeled fatty acids can be used to follow their conversion to other fatty acids and to investigate the biological control exerted on the utilization of deuterium-labeled 9c,12c-18:2, 16:0, 18:0, 9c-18:1 and 12c,15t-18:2.

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EXPERIMENTAL PROCEDURES

Protocol. The two subjects were male Caucasians, ages 24 and 25. Medical histories indicated no congenital ailments or recent medical problems. The subjects had not taken any medication for at least three weeks before the deuterated fats were fed. Weight/height ratios were 24.5 and 23.6 kg/m^2. Blood pressure (120/80 and 117/73), serum cholesterol (145 and 166 mg/dl) and fasting triglyceride (TG) (52 and 58 mg/dl) data were within normal ranges, as were all other clinical blood profile data. This information and a general physical examination confirmed that each subject was in excellent health, that neither suffered from metabolic abnormalities, and that both were representative of the normal population. In addition to these data, the fatty acid compositions of individual phospholipid classes were similar to published values (6–8), and the concentrations of phosphatidylcholine (PC) (1.7 and 1.4 mg/ml) and free fatty acid (0.09 and 0.06 mg/ml) were consistent with reported data (7,9).

Subjects were requested to follow the standard diet recommended for diabetics for one week prior to feeding the mixture of deuterium-labeled fats to aid them in selecting a diet of ca. 40% percent fat, 40% carbohydrate and 20% protein. Dietary histories confirmed that food selection was typical of American diets as reported in the Hanes and USDA surveys (10,11). No detectable changes in the subjects' weights were observed during this period, which indicated a stable energy balance. The subjects were fasted for 10 hr before eating the meals containing the mixture of deuterium-labeled TG.

The weight, identity and location of the deuterium labels for the six deuterium-labeled fatty acids in the TG mixtures are listed in Table 1. The synthesis and purification of these fats have been described previously (12–15). Mixtures of the deuterium-labeled TG were heated to ca. 65 C. A blender was used to emulsify the triglycerides with 30 g calcium caseinate, 30 g dextrose and 15 g sucrose in 200 ml of water, which had been warmed to 65 C. This mixture was fed at 8 a.m. in place of the subjects' normal breakfast. A light lunch at 12:30 p.m. (subject 1) and at 11:45 a.m. (subject 2) and a

normal evening meal at 6:30 p.m. were consumed.

Blood samples (ca. 20 ml each) for plasma lipid class fatty acid analysis were obtained by venipuncture at 0, 2, 4, 6, 8, 12, 15, 24 and 48 hr. Serum samples (ca. 15 ml each) were collected at 2, 4, 6, 8, 12 and 15 hr for isolation of chylomicron TG. The chylomicron TG fractions (S_F 400) were isolated at 23 C from the serum samples by centrifugation at 25,000 rpm for 74 min (5.74×10^6 g-min) in a Beckman SW41 swinging bucket rotor as described previously (16).

TABLE 1

Composition of Triglyceride Mixtures Fed

Deuterium-labeled fatty acid	Subject 1 Wt (g)	Subject 1 Percent	Subject 2 Wt (g)	Subject 2 Percent
16:0-9,10-$^2H_2$	5.30	13.96	6.32	23.40
18:0-9,10,13,13,14,14-$^2H_6$	4.96	13.06	—	—
18:0-9,9,10,10-$^2H_4$	—	—	3.83	14.18
9c-18:1-14,14,15,15,17,18-$^2H_6$	4.71	12.40	5.46	20.23
9c,12c-18:2-15,15,16,16-$^2H_4$	14.39	37.87	4.96	18.36
12c,15t-18:2-9,10-$^2H_2$	8.63	22.70	6.45	23.88
Total weight	37.99		27.02	
Ratios of deuterium-labeled fatty acids in mixtures				
12c,15t-18:2/16:0	1.62		1.02	
12c,15t-18:2/18:0	1.73		1.68	
12c,15t-18:2/9c-18:1	1.83		1.18	
12c,15t-18:2/9c,12c-18:2	0.60		1.30	

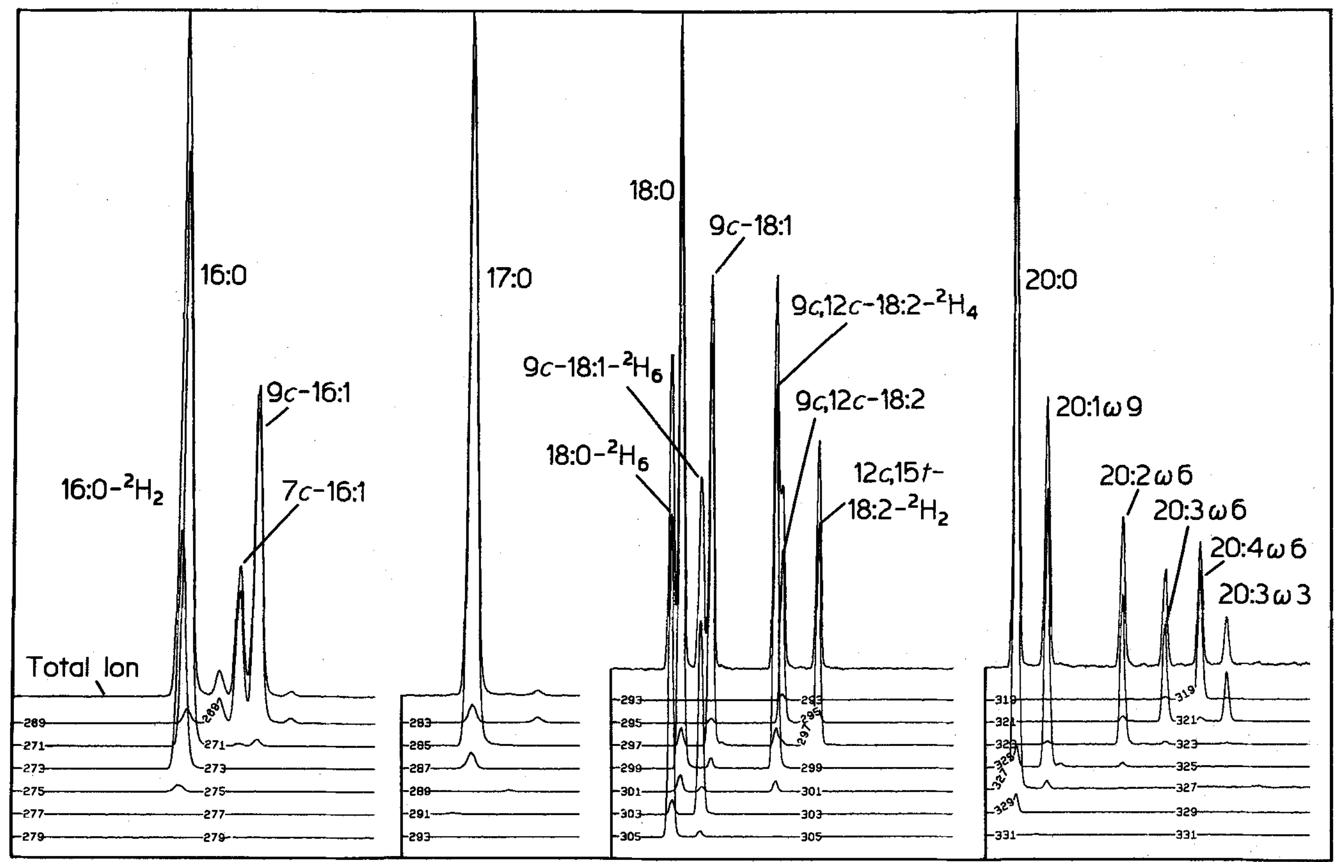

FIG. 1. Gas chromatography (GC)-mass spectrometry chromatogram of a standard mixture containing deuterated and nondeuterated fatty esters. The 30 m × 0.32 mm Supelcowax 10 fused silica GC column was programmed from 165 C to 265 C at 5 C/min. The Finnigan quadrupole mass spectrometer used isobutane to provide chemical ionization mass spectra.

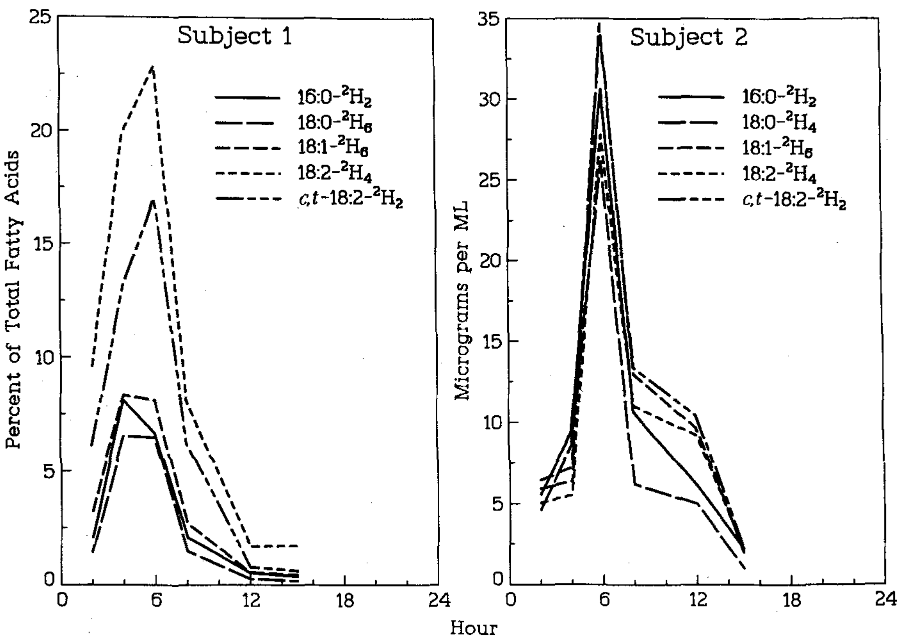

FIG. 2. Incorporation and turnover of deuterium-labeled 16:0, 18:0, 9c-18:1, 9c,12c-18:2 and 12c,15t-18:2 into chylomicron triglycerides. Data for subject 1 are plotted as percent isotopic enrichment of the total fatty acids. Data for subject 2 are plotted as micrograms of deuterated fatty acid per ml of plasma.

TABLE 2

Composition of Chylomicron Triglyceride Samples Containing Maximum Percent Isotopic Enrichment

Deuterium-labeled fatty acid	Subject 1		Subject 2	
	4-hr sample (%)	Fed mixture (%)	6-hr sample (%)	Fed mixture (%)
$16{:}0\text{-}^2H_2$	14.5	13.96	20.0	23.40
$18{:}0\text{-}^2H_6$	11.7	13.06	—	—
$18{:}0\text{-}^2H_4$	—	—	17.0	14.18
$9c\text{-}18{:}1\text{-}^2H_6$	14.9	12.40	22.6	20.23
$9c,12c\text{-}18{:}2\text{-}^2H_4$	35.5	37.87	18.1	18.36
$12c,15t\text{-}18{:}2\text{-}^2H_2$	23.5	22.70	22.4	23.88

[a]Percentages of deuterated fatty acids in chylomicron triglycerides are normalized to 100% for comparison with fed mixture percentages.

Lipid isolation, derivatization and analysis. Total lipids were extracted with 2:1 chloroform/methanol (17), and known weights of triheptadecanoin, cholesteryl heptadecanoate, heptadecanoic acid (17:0) and diheptadecanyl-L-α-phosphatidylcholine were added as internal standards to the total lipid extract to aid in determining actual concentrations (mg/ml) of the deuterated fatty acids in these plasma lipid classes. The free acid of 17:0 was added to the other plasma lipid classes after their isolation by thin layer chromatography (TLC). Internal standards were not added to the chylomicron extracts from subject 1.

Preparative TLC was used for isolation of TG, cholesteryl ester (CE), free fatty acid, phosphatidylethanolamine (PE), PC and phosphatidylserine (PS) lipids (18,19). Methyl esters of the isolated lipids were prepared by heating with a 5% HCl-methanol solution (20). In order to determine the distribution of the deuterium-labeled fatty acids in the 1- and 2-acyl position of PC, a portion of the isolated PC was treated with phospholipase A_2 (21), and the reaction products were separated by TLC and esterified. The methyl esters of these lipid classes were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). A Varian model 3400 gas chromatograph, equipped with a 100 m × 0.25 mm SP2560 fused silica capillary column (Supelco, Bellefonte, Pennsylvania) and a flame ionization detector, was used to analyze many of the samples to confirm the quantitation of the methyl ester data obtained by GC-MS. Operating conditions were split ratio, 1:100; linear veloc-

ity of helium, 21 cm/sec; detector and injection temperature, 235 C. The column oven temperature was linearly programmed from 165 to 220 C at 3 C/min with an initial hold of 15 min and a final hold of 30 min.

Fatty acid percentages and concentrations for both labeled and unlabeled fatty acid esters were obtained by GC-MS analysis with a Finnigan model 4500 quadrupole mass spectrometer operated in a chemical ionization mode with isobutane as the ionization reagent (22). The gas chromatograph-mass spectrometer was equipped with a Supelcowax 10 fused silica column (30 m × 0.32 mm; Supelco) and was temperature programmed from 165 C to 265 C at 5 C/min with a 20-min final hold. A GC-MS chromatogram of a standard mixture is shown in Figure 1. This figure illustrates the monitored ions and the relative retention of the deuterated and nondeuterated fatty acid methyl esters. Fatty acid methyl ester data for chain lengths shorter than 16 and longer than 20 carbons were not collected, so as to minimize GC-MS data collection and procession time. The methyl ester peaks were identified by comparison to GC retention times for known standards and from molecular weight data. Analyses of mixtures of known composition were used to determine the accuracy of the GC-MS data. Analyses of various mixtures of known composition that contained deuterium-labeled and nonlabeled fatty esters were used to simulate the composition of the actual samples. A 5% relative standard deviation was obtained when each of the deuterium-labeled fatty esters was present at 1% of the total sample.

Calculation of selectivity ratio data. Selectivity ratios are calculated according to the following equation: selectivity ratio = experimental ratio/fed ratio, where experimental ratio equals the ratio for two deuterium-labeled fats in a lipid fraction, and the fed ratio equals the ratio for the same two deuterium-labeled fats in the fed mixture. If the value for the selectivity ratio is less than 1.0, the reciprocal is calculated and the value is given a negative sign.

A selectivity ratio with negative sign denotes discrimination against incorporation of the deuterium-labeled fatty acid in the numerator, while a positive selectivity ratio indicates preferential incorporation. The use of the reciprocal for negative selectivity ratios avoids the problem of compressing negative values between 0.0 and 1.0 while allowing the positive selectivity ratios to have a range between 1.0 and infinity. Thus a selectivity ratio of ±1.0 indicates no difference between the ratio of the fats in the sample compared to the ratio in the fed mixtures. Dividing the experimental ratio by the fed ratio adjusts the value of the experimental ratio for unequal amounts of deuterium-labeled fats used in the fed mixtures. This calculation facilitates data comparison from subjects fed mixtures of deuterium-labeled fats containing different ratios.

RESULTS

Absorption of deuterium-labeled saturated and unsaturated fatty acids. The chylomicron TG data in Figure 2

TABLE 3

Comparison of the Relative Incorporation of Deuterium-Labeled Fatty Acids into Human Plasma Lipid Classes

Fatty acid ratio	Subject no.	Selectivity ratios								
		Chylo TG	Plasma							
			TG	CE	FFA	PE	PS	PC	PC-1	PC-2
c,t18:2/16:0	1	1.0	1.2	NV[a]	1.4	1.8	1.5	-1.5	-1.6	3.0
	2	1.1	1.2	NV	-1.4	2.2	1.2	-1.8	1.1	2.4
c,t18:2/18:0	1	1.2	1.6	NV	1.1	-2.5	-6.1	-2.1	-2.3	4.4
	2	-1.1	1.2	NV	-1.6	-3.6	-8.0	-3.2	-2.9	1.6
c,t18:2/18:1	1	-1.2	-1.3	NV	1.1	-1.2	-1.3	2.4	3.1	1.4
	2	-1.2	1.1	NV	1.2	-1.4	-1.7	-2.7	6.7	1.6
c,t18:2/18:2	1	1.1	-1.3	NV	1.4	-3.5	-2.1	-4.0	4.5	-6.3
	2	-1.1	1.1	NV	1.2	-3.5	-2.5	-6.3	8.8	-6.5
18:2/16:0	1	1.1	1.2	7.0	-1.1	3.0	2.7	1.7	-10.5	15.0
	2	1.2	1.4	20.1	1.7	5.5	3.7	3.5	-15.0	20.0
18:2/18:0	1	1.1	1.7	NV	1.3	-1.4	-1.9	1.2	-15.0	27.0
	2	-1.0	1.5	NV	-2.0	-1.3	-2.8	2.0	-25.0	15.0
18:2/18:1	1	-1.2	-1.1	8.7	-1.4	1.6	1.5	5.1	-2.3	9.5
	2	1.1	-1.2	11.9	-1.1	2.4	1.5	15.0	-2.8	11.5
18:1/16:0	1	1.1	1.7	-1.2	1.4	1.9	1.5	-3.8	-4.6	1.6
	2	1.2	1.4	1.7	-1.5	2.3	2.5	-4.8	-5.5	1.5
18:1/18:0	1	1.1	1.8	NV	1.1	-2.4	-4.5	-4.4	-6.6	2.8
	2	1.1	1.5	NV	-1.7	-3.1	-4.2	-8.6	-18.3	1.0
18:0/16:0	1	-1.1	-1.1	NV	1.3	4.5	7.0	1.2	1.4	-1.5
	2	1.2	-1.1	NV	1.2	7.1	10.2	1.8	3.3	1.5

[a]NV: no value, because deuterated 12c,15t-18:2 or 18:0 was not detected. TG, triglyceride; CE, cholesteryl ester; FFA, free fatty acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine.

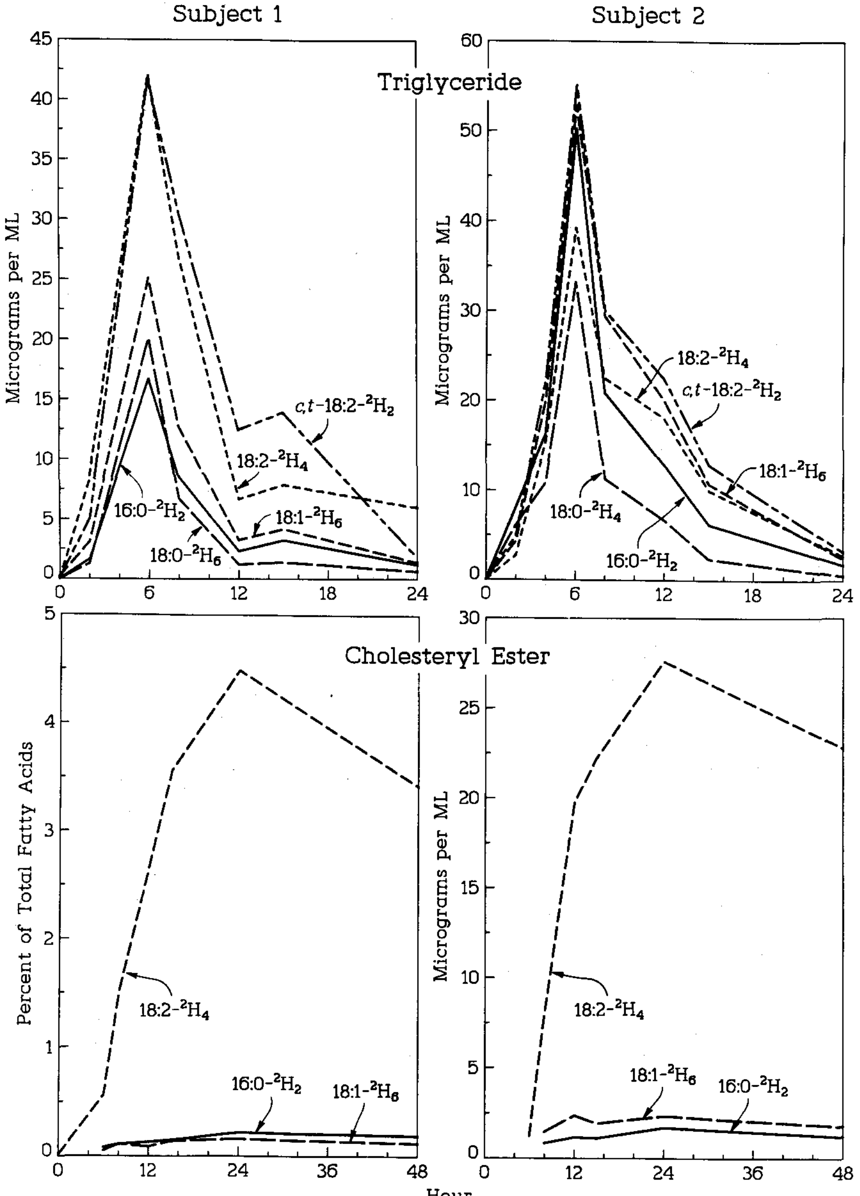

FIG. 3. Uptake and disappearance of deuterium-labeled 16:0, 18:0, 9c-18:1, 9c,12c-18:2 and 12c,15t-18:2 into human plasma triglyceride and cholesteryl ester. Micrograms per ml refer to micrograms of deuterated fatty acid per ml of plasma. Percent of total fatty acids indicates percent isotopic enrichment in the total fatty acids.

are plotted as the percentage of deuterium-labeled fatty acid in the total sample for subject 1 and as micrograms of deuterated fat per ml of plasma for subject 2. The composition of the deuterium-labeled fatty acids in the chylomicron TG samples containing the maximum percent enrichment were normalized to 100% and compared to the composition of the fed mixture in Table 2. Selectivity ratios are given in Table 3 and are based on the total areas under the curves shown in Figure 2. These data are used to reflect preferential removal of one fatty acid relative to another.

The major difference between the chylomicron TG data for the two subjects was the maximum total enrichment. This difference is 23% (61% in subject 1 vs 38% in subject 2). Since previous data for 10 subjects fed various amounts of deuterium-labeled fatty acids (23–27) indicated ~60% maximum total enrichment in chylomicron TG samples, this difference is probably due not to the difference in total amount of deuterium-labeled fatty acid fed but rather to the dilution of deuterated fats in the 6-hr chylomicron sample from subject 2 by fat present in the meal he consumed at 11:45 a.m.

The overall similarity of the curve shapes, combined with the selectivity ratios in Table 3 and the deuterium-labeled fatty acid composition data in Table 2 provides good evidence that absorptions of the various deuterium-labeled fatty acids were essentially equal.

Incorporation of saturated and unsaturated fatty acids into plasma lipids. Plasma TG, CE, PE and PC data are plotted in Figures 3 and 4. These data provide a comparison of the relative incorporation and removal of deuterated 16:0, 18:0, 9c-18:1, 9c,12c-18:2 and 12c,15t-18:2.

Examples of both mg/ml and percent enrichment data for incorporation of deuterium-labeled fatty acids are included. These data show that the relative rate of clearance of 12c,15t-18:2 is similar to other fats commonly present in the diet. Variations in percentages and amounts of the individual deuterium-labeled fatty acids incorporated into the same lipid class for the different subjects are due largely to the difference in percentages of deuterated fats in the fed mixtures. The differences between the various lipid classes from the same subject are due to differences in the selectivity of the enzyme systems involved with the metabolism of these fatty acids.

The selectivity ratios summarized in Table 3 have been adjusted for differences in composition of the fed mixtures. These data reflect the relative incorporation of deuterated fats into the various plasma lipids. The selectivity ratios for 12c,15t-18:2 are plotted in Figure 5 to illustrate the differences between incorporation of this fatty acid relative to 16:0, 18:0, 9c-18:1 and 9c,12c-18:2. From these data and the data in Figures 3 and 4, a number of interrelationships are apparent:

(i) Differences between the selectivity ratios in Figure 5 for the deuterium-labeled fatty acids incorporated into plasma TG and free fatty acid samples are small compared to the differences for the phospholipid fractions. The selectivity ratios for the TG and free fatty acid fractions indicate less than a 1.5-fold difference in the uptake and removal of 12c,15t-18:2 compared to 16:0, 18:0, 9c-18:1 and 9c,12c-18:2. These selectivity ratios indicate that those enzymes involved in metabolism of TG and free fatty acids are relatively insensitive to fatty acid structure and have a minor role in regulation of lipid class fatty acid composition.

(ii) The plasma CE results were unexpected; only 9c,12c-18:2-^{2}H was incorporated to an appreciable extent, and neither 18:0-^{2}H nor 12c,15t-18:2-^{2}H were detected. The enrichment of 9c-18:1-^{2}H and 16:0-^{2}H was also low (0.1–0.2%), as shown in Figure 3. Thus, the selectivity ratios for 18:0-^{2}H and 12c,15t-18:2-^{2}H cannot be calculated, and the selectivity ratios that involve 9c-18:1 and 16:0 have large relative standard errors because of the difficulty of accurately quantitating percent enrichment at the 0.1% level.

(iii) The isotopic enrichment data for 12c,15t-18:2 in Figure 4 and the selectivity ratios plotted in Figure 5 for PE indicate about a two-fold preference for incorporation of 12c,15t-18:2-^{2}H relative to 16:0-^{2}H and an obvious discrimination against 12c,15t-18:2-^{2}H incorporation relative to 18:0-^{2}H and 9c,12c-18:2-^{2}H. Preferential incorporation of 18:0-^{2}H and 9c,12c-18:2-^{2}H into PE is also evident from the selectivity ratios for 9c,12c-18:2 in Table 3 and the isotopic enrichment data in Figure 4.

(iv) The major feature of the PS data is the six- to tenfold preferential incorporation of deuterated 18:0 relative to 12c,15t-18:2. Preferential incorporation of 18:0-^{2}H is reflected by the selectivity ratios in Figure 5 for 18:0-^{2}H vs deuterated 16:0, 9c-18:1 and 9c,12c-18:2. This strong preferential incorporation for 18:0 is the major reason why the 12c,15t-18:2 selectivity ratio for plasma PS is intermediate between 16:0 and 9c-18:1 and clearly different from 18:0.

(v) PC was the only lipid class that appeared to preferentially incorporate 12c,15t-18:2 relative to 9c-18:1. Comparison of the actual isotopic enrichment data in Figure 4 for the various deuterated fatty acids indicate that this positive selectivity ratio for 12c,15t-18:2 vs 9c-18:1 was due to the low incorporation of 9c-18:1 into PC rather than to a selective incorporation of 12c,15t-18:2. The 12c,15t-18:2 vs 9,12c-18:2 selectivity ratio indicates a four- to sixfold preference for incorporation of 9c,12c-18:2. These data are evidence that 12c,15t-18:2 is a poor substrate relative to 9c,12c-18:2 for acylation of PC. The fact that the 12c,15t-18:2 isomer has a methylene-interrupted diene structure but a *trans* double bond in the 15 position (omega-3) further demonstrates the preference of PC acyltransferase for all *cis* polyunsaturated fatty acid structures.

(vi) The small negative selectivity ratios in Table 3 and Figure 5 for 12c,15t-18:2 vs 16:0 and 18:0 indicate that the specificity of PC acyltransferase for 12c,15t-18:2 and saturated fatty acids is similar. The three- to sixfold preferential incorporation of 12c,15t-18:2 relative to 9c-18:1 and 9c,12c-18:2 into the 1-acyl position of PC is similar to the reported differences for saturated and unsaturated fatty acids (28).

(vii) The selectivity ratios in Table 3 and Figure 5 for 2-acylPC reflect the well-known preference for incorporation of 9c,12c-18:2 and exclusion of saturated fatty acids (28). From these selectivity ratios, the specificity of PC acyltransferase for 12c,15t-18:2-^{2}H was closest to 9c-18:1. Comparison of the differences in the various selectivity ratios for the 1- and 2-acylPC positions indicates the possibility of two PC acyltransferases, as suggested by others based on differences in Km values for 1- and 2-acylPC (29,30).

Of interest is the fact that the relative percentages of the deuterium-labeled fatty acid incorporated were not closely related to the overall fatty acid composition of the PC samples. For example, the PC samples contained 24–27% 16:0, 12–14% 18:0, 12–13% 9c-18:1, 29–31% 9c,12c-18:2 and 12–15% 20:4. In comparison, percentages for the deuterium-labeled fats in the PC samples were 10–14% 16:0, 10–19% 18:0, 2–5% 9c-18:1, 50–60% 9c,12c-18:2 and 14–17% 12c,15t-18:2. These data indicate that 9c,12c-18:2 located at the 2-acyl position of PC is exchanged more rapidly than fatty acids at the 1-acyl position of PC by a deacylation-reacylation mechanism, but the overall fatty acid composition is not greatly altered.

The data in Table 3 allow a comparison of all the possible combinations of selectivity ratios for the various fatty acids in the fed mixtures. Since the selectivity ratios are corrected for differences in the fatty acid content of the fed mixtures, they should be the same. Thus, differences in the selectivity ratios for lipid fractions from subject 1 and subject 2 may provide insight into how the ratio of the various fatty acids in the diet influences the relative incorporation of each of the fatty acids. Support for this suggestion is provided by the relatively close agreement of the TG selectivity ratios for the two subjects and the large differences for many of the phospholipid selectivity ratios. Small differences of ± 0.4, such as those for the plasma TG ratios, can be attributed to accumulation of experimental or analytical errors. Differences larger than ± 0.4 reflect differences that could be due to the composition of the deuterated fat mixture. The 9c,12c-18:2/16:0 selectivity ratios for PE and PC indicate incorporation of about twice as much 9c,12c-18:2 as 16:0 for subject 2, who received the low 18:2 diet. Similarly, the

9c,12c-18:2/9c-18:1 ratio is three times higher for plasma PC from subject 2, and the 18:2/16:0 and 18:2/9c-18:1 ratios are both much higher for the CE fraction from subject 2. These data suggest that 9c,12c-18:2 is incorporated more selectively when 9c,12c-18:2 is present at lower concentrations in dietary fat. However, the validity of this suggestion remains to be confirmed because available data is limited.

A similar trend is noted for the 12c,15t-18:2 selectivity ratios plotted in Figure 5; the ratios for the phospholipid classes were generally more extreme for the subject fed the mixture containing the lower amount of 9c,12c-18:2. Subject 1 received 2.9 times more deuterium-labeled 9c,12c-18:2 on a weight basis than subject 2, but the amount of 9c,12c-18:2 incorporated into PC and PE samples from subject 1 was 20–100% less than expected compared to the PC and PE data for subject 2. Thus, for the subject receiving the mixture with the higher deuterium-labeled 9c,12c-18:2 content, relatively less deuterium-labeled 9c,12c-18:2 was incorporated compared to deuterium-labeled 12c,15t-18:2.

These data are consistent with animal data that indicate if the diet is high in 9c,12c-18:2, there are control mechanisms that increase the β-oxidation rates for 9c,12c-18:2 (31) and limit incorporation of 9c,12c-18:2 into phospholipids (32–34). These mechanisms are apparently available to control phospholipid fatty acid composition within relatively narrow ranges in the presence of dietary fat with wide variation in fatty acid composition. The important implication of these data is that a diet with a high polyunsaturate/saturate ratio will increase the 9c,12c-18:2 content of phospholipids less than would be

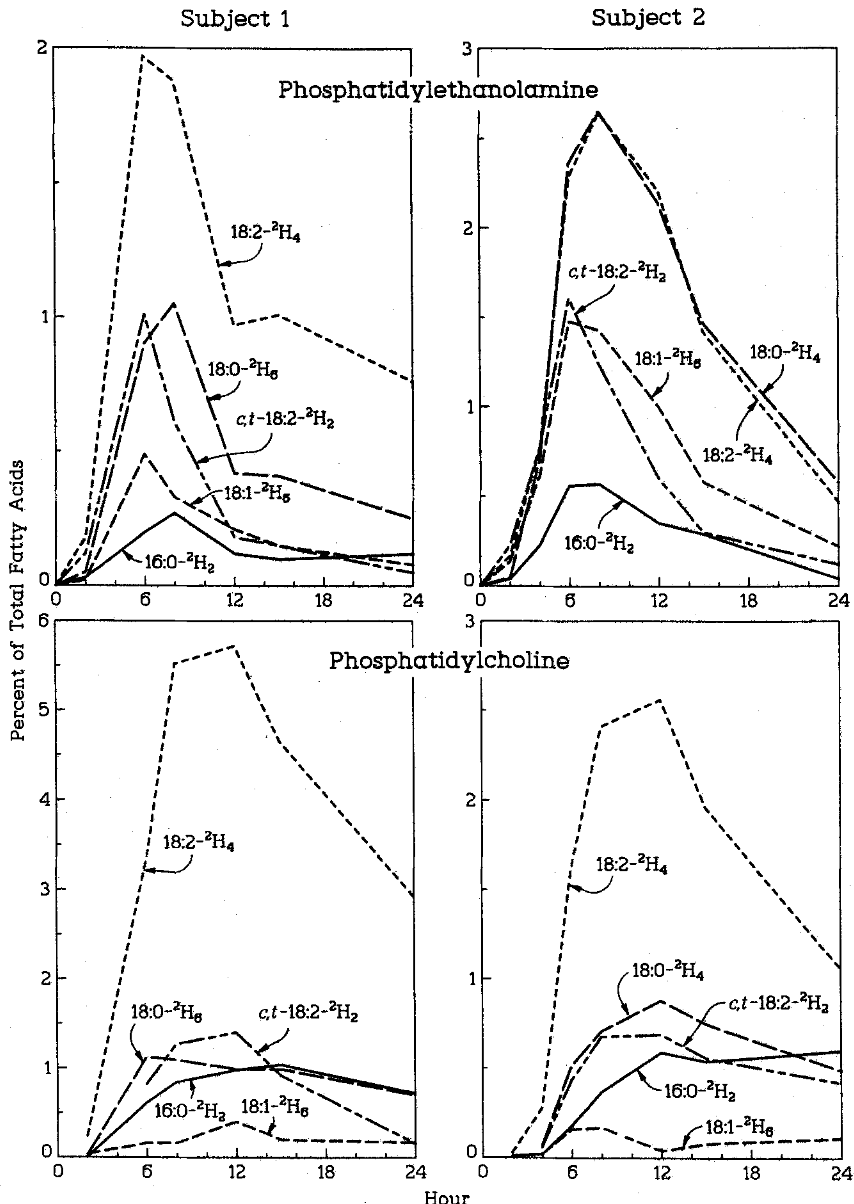

FIG. 4. Uptake and disappearance of deuterium-labeled 16:0, 18:0, 9c-18:1, 9c,12c-18:2 and 12c,15t-18:2 into human plasma phosphatidylethanolamine and phosphatidylcholine. Percent of total fatty acids indicates percent isotopic enrichment in the total fatty acids.

expected compared to a diet with a low polyunsaturate/saturate ratio.

Conversion of deuterium-labeled fats. The major fatty acids present in the plasma lipid classes were examined for the presence of deuterium-labeled fatty acids other than those in the fed mixtures. None of the lipid classes contained detectable amounts (above .01% enrichment) of deuterium-labeled fatty acid other than those fed.

DISCUSSION

Since only two subjects were studied, the data cannot be statistically evaluated as representative of the normal population. However, based on clinical data and medical histories, we believe the metabolism of these deuterated fatty acids is typical for young-adult males. This conclusion is supported by plasma cholesterol, TG and phospholipid concentrations and by fatty acid composition data (not shown) for the various lipid classes.

Our experimental design allows each subject to serve as his own control. This permits direct comparison of the incorporation of each of the five deuterated fatty acids in individual subjects. This design also negates many of the confounding problems of biological variation.

Absorption. The chylomicron TG data in Figure 2 and Table 2 are evidence that the relative absorptions of the deuterium-labeled fatty acids were similar. The only difference was a slight skew of the saturated vs unsaturated fatty acid curves, which suggests a slightly more rapid absorption of 16:0 and 18:0. The chylomicron TG data are plotted in Figure 2 as percentage of each deuterated fatty acid in the total fatty acids for subject 1 and as micrograms of each deuterated fatty acid per ml of serum for subject 2. These data show that either form of representing the data provides plots that are suitable for assessing the relative absorption of the deuterated fatty acids. Plotting the data as micrograms of deuterated fatty acid per ml of plasma yields sharper curves because the amount of non-deuterium-labeled endogenous TG in the chylomicrons increases as the deuterated fatty acids are being absorbed.

Absorption of the deuterium-labeled 18:0 and unsaturated fatty acids was similar, which was unexpected because other studies have reported that trioctadecanoin is poorly absorbed (35–37). The only explanation we can propose for the difference between these data and previous results is that care was taken to ensure that the deuterium-labeled trioctadecanoin, which melts above body temperature, was well emulsified by mixing at temperatures above the melting point of trioctadecanoin, which permits even distribution on to the caseinate. This procedure would be expected to prevent formation of crystalline particles of trioctadecanoin, which would be resistant to hydrolysis by pancreatic lipase.

The presence of non-deuterium-labeled fatty acids in the chylomicron TG samples is attributed to incorporation of endogenous TG during formation of the chylomicron particles. The value of about 40% non-deuterium-labeled fatty acid in the 4- to 6-hr chylomicron TG samples has been a consistent feature of all of our previous chylomicron TG data (23–27). It is also in agreement with rat data, which indicated that as much as 50% of the fat incorporated into chylomicron TG is from an endogenous source (38).

The mixing of dietary and stored or endogenous fat alters the ratio of fatty acids in the chylomicron TG prior to their subsequent metabolism. The implication is that this process may be an important step in the modulation of dietary fatty acid composition and is a mechanism by

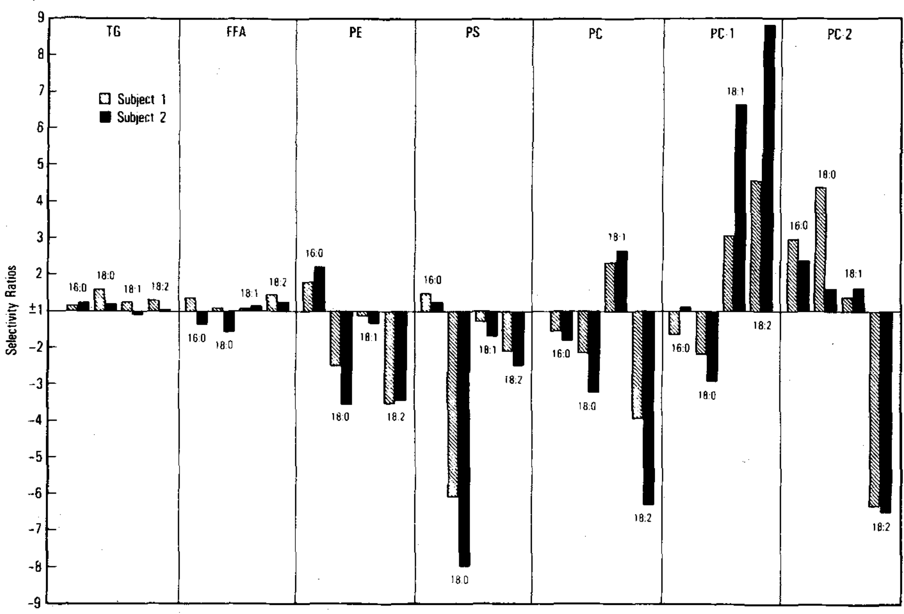

FIG. 5. Selectivity ratios for comparison of the incorporation of deuterium-labeled 12c,15t-18:2 relative to 16:0, 18:0, 9c-18:1 and 9c,12c-18:2 into plasma triglyceride (TG), free fatty acid (FFA), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), 1-acylphosphatidylcholine (PC-1) and 2-acylphosphatidylcholine (PC-2).

which the body partly compensates for wide variation in dietary fatty acid composition.

Interconversion. In this study, interconversion products such as deuterium-labeled 16:1, 18:0, 18:1, 20:3 and 20:4, were not detected in any of the lipid classes. The GC-MS methodology was capable of qualitatively detecting these deuterium-labeled fatty acids at a 0.04% enrichment level. The PC samples were, in particular, carefully analyzed for 20:4 because deuterium-labeled 20:4 should be concentrated in PC. The percentage of 20:4 in the PC samples from these subjects varied between 10 and 13% and the concentration of PC was between 1.4 and 1.7 mg/ml. Since the concentration of 20:4 in PC is ca. 0.15 mg/ml, we calculate that, if a concentration of ca. 60 ng/ml of deuterium-labeled 20:4 were present in the PC samples, we would have been able to detect it. Based on a total blood volume of ca. 3000 ml, only 0.18 mg of the 15 g (subject 1) and 5 g (subject 2) of 18:2-$^2H_4$ fed would have needed to be converted to 20:4-$^2H_4$ to be detected. This amount corresponds to a conversion of 0.0012% (subject 1) and 0.0036% (subject 3) of the 18:2-$^2H_4$ fed. The absence of detectable levels of 20:4 indicates that the rate of conversion of 18:2-$^2H_4$ to 20:4-$^2H_4$ is extremely low in normal subjects.

From these data, it appears that if various deuterium-labeled fatty acids and their precursors are fed in the same mixture, interconversions of one deuterated fatty acid to another will not cause a major problem with the quantitation of the deuterated fatty acids in the fed mixture. For example, if 20:4-$^2H_4$ and 18:2-$^2H_4$ are both fed, the conversion of 18:2-$^2H_4$ to 20:4-$^2H_4$ is so small that the endogenously formed 20:4-$^2H_4$ will not cause an error in the measurement of exogenous 20:4-$^2H_4$ incorporated into plasma lipids. In a similar manner, these data indicate that the rates of conversion of exogenous deuterated 18:0 to 9c-18:1 and of deuterated 16:0 to 18:0 and c-16:1 are low. These results indicate that fatty acid interconversion rates are generally too low to be followed by this particular experimental approach, although in earlier human studies with deuterium-labeled 18:1 isomers, conversion of 16:1 was observed (26,27). The reason for the lack of detectable deuterium-labeled fatty acid interconversion products may be a combination of low elongation and desaturation rates and a slow, gradual release of the products from the liver into the plasma.

Turnover and incorporation of deuterium-labeled fatty acids in plasma lipids. The plots in Figures 3 and 4 for TG, CE, PC and PE graphically show that turnovers of all of the deuterated fatty acids were similar, irrespective of whether a specific fatty acid was selectively incorporated. The data indicate that differences in the selectivity ratios plotted in Figure 5 are due mainly to enzyme selectivities associated with incorporation rather than to differences in turnover rates. The times at which maximum isotopic enrichment occurred in the various lipid classes were consistent for lipid classes from both subjects. The examples given are for data plotted as micrograms of each deuterated fatty acid per ml of plasma and as percentage of each deuterated fatty acid present in the total fatty acids. Examples of both types of plots are shown to demonstrate that the variation in levels of isotopic enrichment in these fractions is reflected on both a percentage and a concentration basis. The similarity between the data from the two subjects was generally good. However, note that almost three times more 9c,12c-18:2-^2H was fed to subject 1 than to subject 2 and that the 18:2 selectivity ratios for PC and PC-1 in Figure 5 for subject 1 are lower than for subject 2. This difference suggests that the composition of the dietary fatty acids has some influence on the amount of each individual fatty acid incorporated. The influence of the dietary fatty acids is most obvious if the low isotopic enrichment (ca. 0.5%) for 9c-18:1-$^2H_6$ in the PC fraction shown in Figure 4 is compared to PC data from previous studies where 9c-18:1 was fed with the *cis* and *trans* isomers of 10- and 11-18:1 (26,27). In these studies, three to four times as much (1.5–2.0%) 9c-18:1-^2H was incorporated.

The importance of dietary 9c,12c-18:2 is evident from the plasma CE data in Figure 3. The proportions of deuterated 9c,12-18:2, 9c-18:1 and 16:0 incorporated into the plasma CE fraction are much different than the fatty composition normally reported for CE. This suggests that dietary 9c,12c-18:2 is utilized preferentially relative to endogenous 9c,12c-18:2 for esterification of cholesterol.

Plasma lipid selectivity ratios. The selectivity ratios are calculated from the areas under the curves after the data are plotted, as shown in the examples in Figure 3 and 4. The selectivity ratios are thus, in effect, weighted averages of several data points. Overall, the selectivity ratios depicted in Table 3 and Figure 5 indicate a strong biological control of fatty acid utilization in healthy males and a general discrimination against incorporation of 12c,15t-18:2 into plasma phospholipids. For example, the incorporation of 12c,15t-18:2 into total PE, PS and PC is decidedly discriminated against compared to 9c,12c-18:2 and 18:0, but 12c,15t-18:2 is preferentially incorporated into PE and PS relative to 16:0. Most of the selectivity ratios for the phospholipids are negative, and there are no large positive values for the neutral lipids, suggesting that the 12c,15t-18:2 isomer was utilized mainly for energy. The lack of any large positive selectivity ratio for 12c,15t-18:2 was consistent with the failure to detect this isomer in human tissue lipid extracts (39). Thus, based on these selectivity ratios and previous tissue fatty acid composition data, it appears unlikely that 12c,15t-18:2 has any significant impact on cell membrane function or on cell enzyme activities at the low levels present in hydrogenated fats.

In addition, the selectivity ratio and isotopic enrichment data imply that for a high intake of dietary 9c,12c-18:2, excess 9c,12c-18:2 is diverted into either storage or β-oxidation pathways, and the influence of a diet high in 9c,12c-18:2 on the 9c,12c-18:2 content of phospholipids is not linear with the increase in the polyunsaturate/saturate ratio once cell and membrane requirements for 9c,12c-18:2 are met.

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Lynne Copes and Sandy Duval provided technical assistance, and D.J. Wolf provided computer programming for data collection and processing.

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Tamoxifen-Induced Modification of Serum Lipoprotein Phospholipids in the Cockerel

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The administration of tamoxifen (Tam), a nonsteroidal antiestrogen, or of a diphenylmethane derivative of Tam that does not bind to the estrogen receptor (DPPE) of cockerels results in a marked decrease in the concentration of serum lipoprotein constituents with an apparent alteration in phospholipid composition. To establish the nature of changes in phospholipids, the molecular species of phosphatidylcholine (PC) and sphingomyelin (Sph) were isolated and characterized. Between 9 and 18 hr following the administration of Tam or DPPE, there was a marked decrease in the proportion of molecular species of serum PC containing C16 and C18 fatty acids, but there was an increase in the proportion of molecular species containing C20 and C22 polyunsaturated fatty acids. Fatty acid analyses revealed that this change was due to an increase in arachidonic and docosahexaenoic acids at the expense of oleic and linoleic acids. These proportional changes were due to an absolute decrease in serum of PC molecular species containing palmitic and stearic acids in association with oleic and linoleic acids with very little change in the absolute concentration of molecular species containing arachidonic and docosahexaenoic acids. By contrast, the composition of Sph, which contained palmitic acid as the major fatty acid, was not altered during treatment. It is concluded that the short-term effect of Tam and DPPE on plasma phospholipids of the cockerel is due to a selective conservation of PC containing long chain polyunsaturated fatty acids.
Lipids 22, 505–512 (1987).

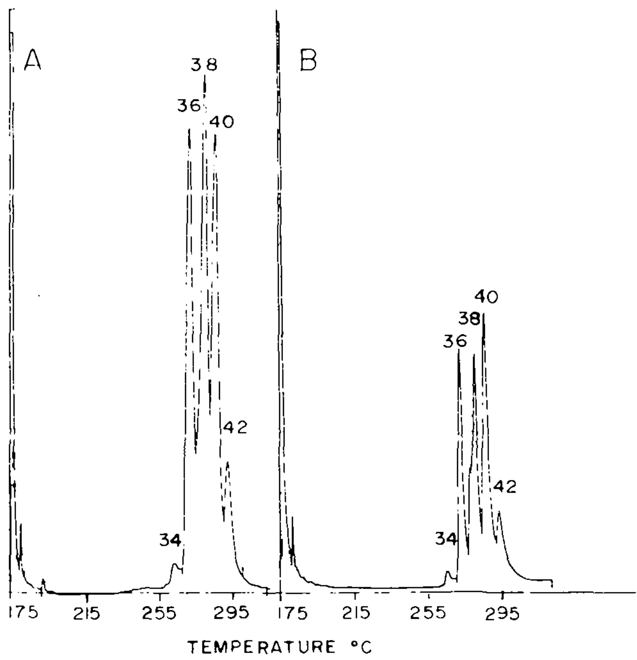

FIG. 1. Gas liquid chromatographic profile of trimethylsilylethers of diacylglycerols from phosphatidylcholine of cockerel serum. (A) Control birds fed ad libitum and injected with propyleneglycol. (B) Birds fed ad libitum and injected with Tam 9 hr prior to blood sampling. Carbon numbers are representative of the retention time equivalent to triacylglycerol with the respective number of acyl carbon atoms.

Phospholipids constitute a major lipid class in plasma, where they function to package neutral lipids in the lipoproteins (1) and to maintain amphipathic helices in apoproteins (2,3), which function as activators of key enzymes in lipoprotein metabolism or as cellular receptor recognition sites (4) for removal of lipoproteins from the circulation. A wide diversity of molecular species of phospholipids is found in plasma lipoproteins (5–7). Although it is generally considered that exchange/transfer proteins promote the equilibration of phospholipid molecular species among lipoproteins (8), there is clear evidence that sphingomyelin (Sph) does not equilibrate completely among lipoproteins (9), while there is a tendency for the more polyunsaturated phosphatidylcholines (PC) to be preferentially associated with high density lipoproteins (HDL) (7,10,11). There is little information concerning the importance of specific phospholipid molecular species in influencing the biological activity of apolipoproteins. Indeed, most studies of apolipoprotein function have been conducted with relatively saturated phospholipids (2–4), which may confer biophysical characteristics on the apolipoprotein structure that are distinct from those of phospholipids containing polyunsaturated fatty acids.

The turnover of plasma phospholipids among plasma lipoproteins has not been extensively studied due to the rapid equilibration of label among lipoproteins. It is generally believed that a major portion of the phospholipids enters the plasma compartment with chylomicrons and very low density lipoproteins (VLDL). During lipolysis of triacylglycerols, a portion of the phospholipid is shed to HDL while another portion, which is relatively enriched in Sph, moves with apo B to low density lipoproteins (LDL) (12–14). The mechanisms for control of the molecular species composition of plasma PC are poorly understood, but dietary fatty acids (7,15) and selective metabolic pathways for PC biosynthesis in the liver (1) may play a role.

As part of an investigation into the effects of nonsteroidal antiestrogen agents on cockerel serum lipoproteins, we noted a rapid alteration in the pattern of phospholipid molecular species of lipoproteins (Lazier, C.B, and Breckenridge, W.C., submitted for publication). The present investigation was undertaken in

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Abbreviations: Tam, tamoxifen; DPPE, N,N-diethyl-2[(4-phenylmethyl)-phenoxy]ethanamine; PC, phosphatidylcholine; Sph, sphingomyelin; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; PE, phosphatidylethanolamine.

order to determine the exact effect of these agents on the composition of plasma lipoprotein phospholipids.

MATERIALS AND METHODS

Animals and injections. Male chickens (White Leghorn, 500–600 g) were hatched in local facilities and kept in quarters lighted for 12 hr daily. Food (standard chicken chow) and water were given ad libitum, except as noted in the text.

Tamoxifen (Tam) and N,N-diethyl-2[(4-phenylmethyl)-phenyoxy]ethanamine (DPPE) HCl were prepared at a concentration of 25 mg/ml in propyleneglycol. Tam (as the citrate) was obtained from Sigma Chemical Co. (St. Louis, MO). DPPE was a gift from Dr. Lorne Brandes (University of Manitoa, Winnipeg, Manitoba, Canada), who prepared it by the procedure given in ref. 16. The birds were injected at a dose of 100 mg/kg body weight. Controls or fasting birds were injected with propyleneglycol.

Injections of Tam citrate and DPPE in propyleneglycol or of the vehicle alone were given into the breast muscle. For the 9-hr treatment, the injections were made at 08:00 and the animals were killed at 17:00. For the 18-hr treatment, injections were routinely given at 16:00, and the animals were killed at 10:00 the next day. The lights were turned off from 19:00 to 07:00. For initial studies at 4 hr and 48 hr, the animals were injected at 10:30 and 16:00, respectively.

Preparation of serum and lipoprotein fractions. Serum was prepared from trunk blood, which was allowed to clot for 1 hr at 2 C and then was centrifuged for 20 min at 15,000 × g. Lipoproteins were isolated for 3–5 ml of serum by ultracentrifugation (17) in a Beckman 50:3 rotor under the following conditions: (a) VLDL at a density of 1.006 g/ml at 40,000 rpm for 18 hr; (b) LDL between a density of 1.006 and 1.063 g/ml at 40,000 rpm for 20 hr; (c) HDL between a density of 1.063 and 1.21 g/ml at 40,000 for 24 hr. The lipoprotein fractions were collected in 1–2 ml portions, dialyzed against saline and analyzed for lipid composition.

Analysis of lipids. The lipids in the lipoprotein fractions were quantitated by gas liquid chromatography of total lipid profiles as described elsewhere (8). In brief, phospholipase C, which converts PC, lysoPC and Sph to diglyceride, monoglycerides and ceramides, respectively, was added to aliquots of serum lipoproteins containing 50–300 mg of lipid along with the Tris buffer (pH 7), calcium chloride (1.2 ml of 10% solution) and ethyl ether (1 ml). After digestion under vigorous agitation, an internal standard (tridecanoin) was added in chloroform, and the lipids were partitioned in chloroform/methanol according to the method of Folch et al. (19). The choloroform was evaporated, and the lipid residue was treated with Trisil BSA Formula P (50–300 ml) to silylate hydroxyl groups. The lipid profiles were analyzed in an automated Hewlett-Packard 5840 gas chromatograph using nickel columns (1/8 × 20 in.) packed with 3% OV-1 on Gas Chrom Q (Applied Sciences Lab). The columns were programmed from 170 to 350 C. Response factors were established for each component by analysis of standards. For analysis of molecular species, PC and Sph were purified by thin layer chromatography, converted to diacylglycerols and ceramides, respectively, by digestion and phospholipase C and then analyzed as trimethylsilyl ethers (9,13). The fatty acids of isolated PC and Sph were analyzed by gas liquid chromatography of the methyl esters following transmethylation of the phospholipid with 6% w/v H_2SO_4 in anhydrous methanol (9). The analysis was completed on a 15-m fused silica column containing DB-225 as the liquid phase at a film thickness of 1 μ. Conditions of operation were column temperature, 195 C; injection temperature, 225 C; detector temperature, 250 C; and carrier gas N_2 at 10 ml/min, H_2 at 15 ml/min, and air at 250 ml/min. Peaks were identified by retention time based on chromatography with standard fatty acids or for minor peaks by plots of relative retention times.

RESULTS

Tam has been used extensively as an antiestrogen agent because of its ability to bind competitively to the estrogen receptor. In addition to this characteristic, it also binds with high affinity to a poorly characterized site in microsomal membranes of liver (antiestrogen binding sites). Recent studies (Lazier, C.B., and Breckenridge, W.C., submitted for publication) showed that Tam

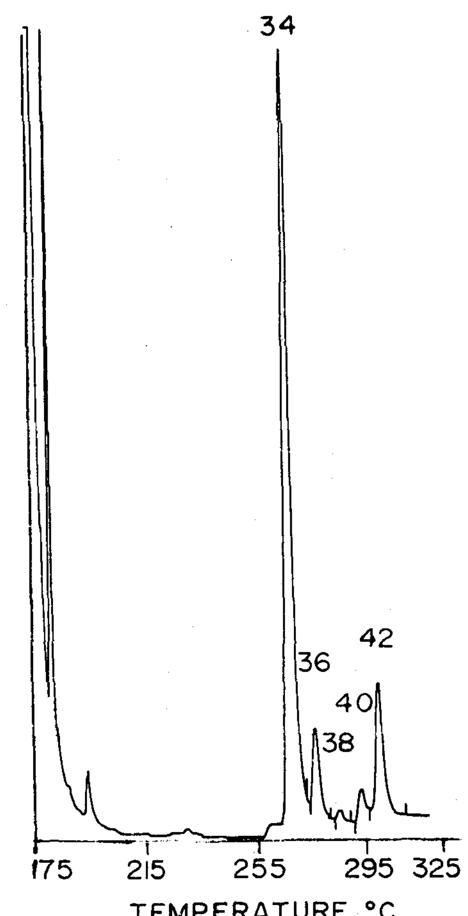

FIG. 2. Gas liquid chromatographic profile of trimethylsilyl ethers of ceramides from Sph of cockerel serum. Carbon numbers are representative of the retention time equivalent to triacylglycerol with the respective number of acyl carbon atoms.

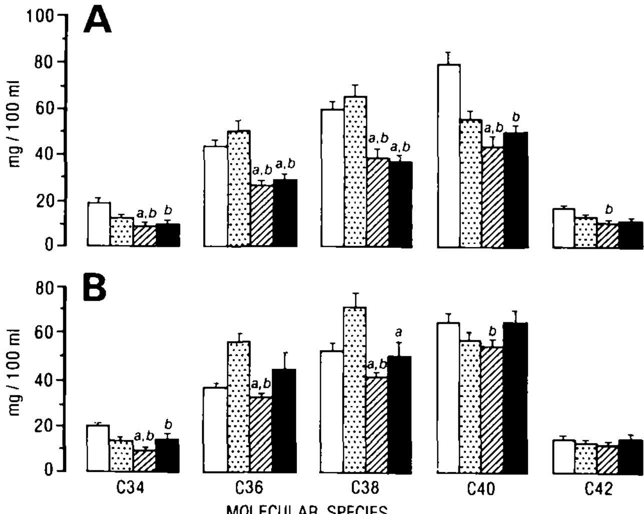

FIG. 3. Absolute concentrations of phospholipid molecular species in cockerel serum. A and B are 9 and 18 hr, respectively, after injection of propyleneglycol with fasting (open column); propyleneglycol with ad libitum feeding (dotted column); Tam with ad libitum feeding (hatched column); and DPPE with ad libitum feeding (solid column). *a*, Significantly different (p <0.01) from propyleneglycol control, fed ad libitum. *b*, Significantly different (p <0.01) from fasted control.

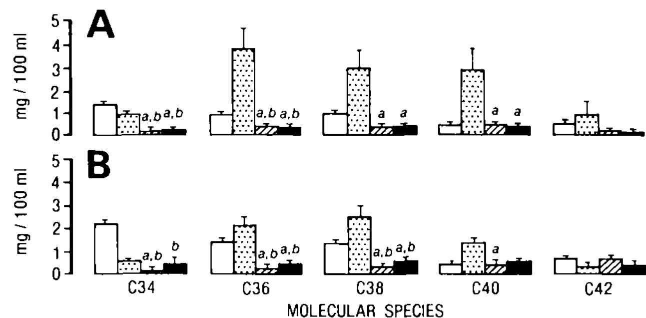

FIG. 4. Absolute concentrations of phospholipid molecular species in cockerel serum very low density lipoprotein. A and B are 9 and 18 hr, respectively, after injection of propyleneglycol with fasting (open column); propyleneglycol with ad libitum feeding (dotted column); Tam with ad libitum feeding (hatched column); and DPPE with ad libitum feeding (solid column). *a*, Significantly different (p <0.01) from propyleneglycol control, fed ad libitum. *b*, Significantly different (p < 0.01) from fasted control.

and a derivative of Tam (DPPE), which does not bind to the estrogen receptor but binds to the antiestrogen binding site, lower plasma cholesterol, triacylglycerol and phospholipid concentrations. As shown in Table 1, the administration of Tam and DPPE to cockerels results in a marked suppression of LDL and HDL lipid mass at 9 hr. Although the Tam also suppresses lipid mass at 18 hr, the effect of DPPE disappears by this time. Neither drug gave an effect at 4 hr, while the effect of Tam disappears by 48 hr (data not shown). Although fasting and Tam and DPPE treatment all reduce VLDL, there is little influence of fasting on LDL and HDL concentrations. DPPE but not Tam causes a small reduction in food consumption by the birds at 9 hr (Lazier, C.B., and Breckenridge, W.C., submitted for publication).

Although there is a large decrease in lipoprotein mass, there is a relatively slight change in lipoprotein composition. After 9 hr of treatment with Tam and DPPE, LDL was slightly richer in cholesteryl ester at the expense of triacylglycerol compared to the control (propyleneglycol) or fasting (Table 2). At 18 hr this effect was absent. The proportion of free cholesterol to phospholipid is lower in fasted samples (9 hr, 0.220; 18 hr, 0.241) than in samples from propyleneglycol (0.336 and 0.321), Tam (0.336 and 0.382) and DPPE (0.411 and 0.371) treatment. There is a slight increase in cholesteryl ester at the expense of triacylglycerol in HDL from Tam or DPPE treatment at 9 hr compared to controls (Table 3). The relative concentration of phospholipid is slightly lower in the fasted samples compared with the other treatments, while the cholesterol/phospholipid ratio of HDL was slightly lower (0.059–0.069) for the fasted birds than for the other three treatment groups (0.074–0.091). Thus, these data indicate that a large decrease in plasma triacylglycerol influences the triacylglycerol content in LDL as well as HDL. The alteration in the cholesterol/phospholipid ratio appears to be influenced by fasting more than by the other treatments.

Previous studies had suggested a change in the molecular species composition of the phospholipids (Lazier, C.B., and Breckenridge, W.C., submitted for publication). To investigate this aspect in more detail, PC and Sph were resolved by thin layer chromatography and analyzed for molecular species composition. Qualitative assessment of the thin layer plates revealed that PC and Sph were the major phospholipids, with small amounts of lysoPC and phosphatidylethanolamine (PE). The latter two phospholipid classes were not investigated further. The molecular species of PC are resolved (Fig. 1) into five carbon number groups consisting of C34 (16,16 fatty acid composition), C36 (16,18), C38 (18,18 and 16,20), C40 (18,20 and 16,22) and C42 (17,21). The carbon number identifies the retention time of the trimethylsilyl ethers in relation to tricylglycerols with the designated number of total acyl carbon atoms. There is a substantial increase in peaks 40 and 42 relative to 36 and 38 in the PC from Tam-treated birds compared with the propyleneglycol treatment. The proportion of C40 and C42 in HDL is higher in Tam- or DPPE-treated birds than in propyleneglycol controls (Table 4). The proportion of long chain molecular species is also higher in fasting conditions. It is important to note that the molecular species in LDL and HDL from propyleneglycol-treated birds are very similar in LDL and HDL. However, in fasting conditions and in treatment with Tam and DPPE, the HDL have larger proportions of C40 and C42 than LDL. The increase in the long chain molecular species is associated primarily with an increase in the proportion of arachidonic and docosahexaenoic acids (Table 5) at the expense of oleic and linoleic acids. The amount of palmitic and stearic acids is a relatively constant in all samples.

Sph molecular species (Fig. 2) contain C34 as the major component (60–70%), with C42 as the other significant constituent. The carbon number distribution in control animals for LDL is C34 (73%), C36 (8.8%), C38 (1.2%), C40 (2.8%) and C42 (14.2%), while HDL has C34 (59.2%), C36 (10.2%), C38 (3.9%), C40 (4.3%) and C42 (22.0%). This distribution is due to a high

content of palmitic (60–70%) and lignoceric acids (10–20%), with minor amounts of stearic, oleic, arachidic and behenic acids accounting for the other fatty acids. There is no extensive change in the Sph composition during treatment.

The results clearly show a marked change in the composition of the molecular species of PC, in which C36 and C38 are decreased in relation to C40 and C42. To assess whether the change in these proportions was due to a decrease in the absolute amounts of C36 and C38, the total amount of phospholipid molecular species was determined from the total lipid profiles for serum (Fig. 3), VLDL (Fig. 4), LDL (Fig. 5) and HDL (Fig. 6) for each treatment group (5–9 birds per treatment or time). Each peak is the sum of the mass of diacylglycerol and ceramides for the respective carbon number. For example, C34 represents the palmitoyl ceramide peak of Sph and cochromatographs with C34 diacylglycerol from PC. The makeup of each peak was estimated from the analysis of the ceramides from Sph and diacylglycerols from PC (Figs. 1 and 2, Tables 4 and 5) and distribution of these components in relation to the composition of the phospholipid in the total lipid profile. In the total lipid profile it was calculated that C34 was derived largely from ceramide (80%), while peaks C36, C38 and C40 were 90%, 97% and 95% diacylglycerol, respectively. C42 is a mixture of ceramide and diacylglycerols. Thus the changes in the absolute amounts of C36, C38 and C40 in the total lipid profile are a good estimate of changes in the absolute amounts of PC with these carbon numbers.

Nine hr following treatment with Tam or DPPE, there was a 40–50% decrease in the absolute mass of serum phospholipids (Fig. 3) of the molecular species C36 and C38 compared to the propyleneglycol controls or fasting birds. There is a small decrease in C40 and no change in C42. This effect persists for Tam at 18 hr but has disappeared for DPPE. Thus the enrichment in long chain molecular species of serum PC noted in Table 3 is due to a selective decrease in the absolute amount of molecular species containing C16 and C18 fatty acids. On the basis of the fatty acid composition and the molecular species profile, it is concluded that this loss is due to a decrease of molecular species containing palmitic or stearic acid in combination with oleic or linoleic acid.

The content of all molecular species in VLDL is extremely low due to the virtual elimination of VLDL by Tam or DPPE (Fig. 4). There is about a 50% reduction in C34, C36, C38 and C40 molecular species in LDL at 9 hr for both DPPE and Tam (Fig. 5). Thus, there is a general reduction in all phospholipid classes in keeping with a reduction in LDL mass. While the DPPE effect disappears by 18 hr, the Tam effect persists at 18 hr for C34, C36 and C38 but is eliminated by 48 hr (data not shown). Tam and DPPE treatment cause a pronounced decrease of C36 and C38 of HDL in comparison to propyleneglycol-treated controls (Fig. 6). Fasting tends to cause an increase in C40 compared with all other treatment groups. The effect of Tam and DPPE is apparent at 9 and 18 hr. Since HDL comprises a much larger portion of the total plasma phospholipid than LDL, it essentially determines the overall composition noted for total serum. The concentration of HDL phospholipids is higher in fasting compared to the other treatments for all the major molecular species at 9 hr. At 18 hr the absolute amounts tend to be lower than the propyleneglycol control and similar to Tam and DPPE.

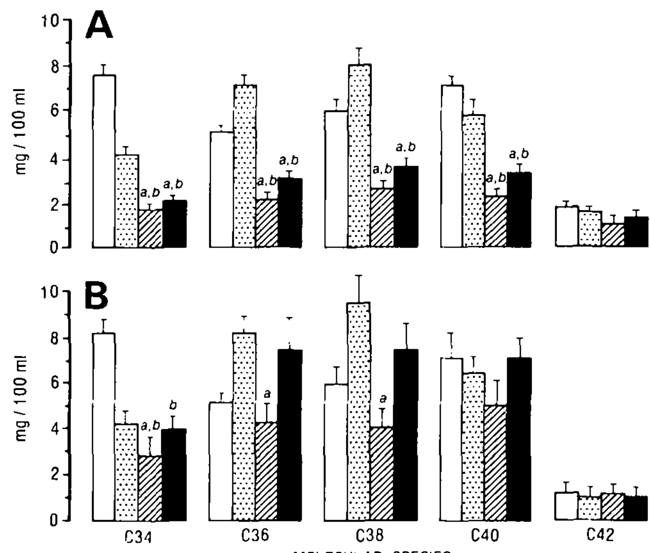

FIG. 5. Absolute concentrations of phospholipid molecular species in low density lipoprotein. A and B are 9 and 18 hr, respectively, after injection of propyleneglycol with fasting (open column); propyleneglycol with ad libitum feeding (dotted column); Tam with ad libitum feeding (hatched column); and DPPE with ad libitum feeding (solid column). *a*, Significantly different (p <0.01) from propyleneglycol control, fed ad libitum. *b*, Significantly different (p <0.01) from fasted control.

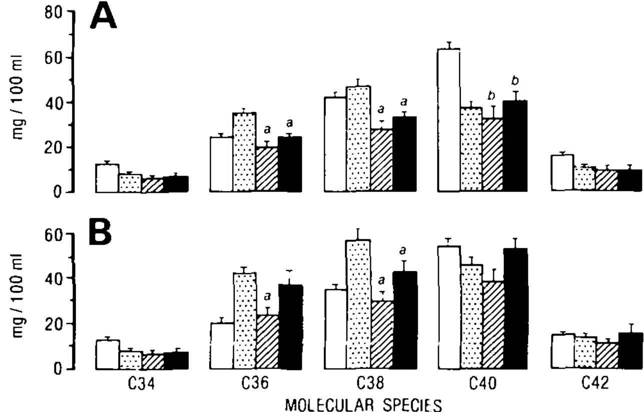

FIG. 6. Absolute concentrations of phospholipid molecular species in high density lipoprotein. A and B are 9 and 18 hr, respectively, after injection of propyleneglycol with fasting (open column); propyleneglycol with ad libitum feeding (dotted column); Tam with ad libitum feeding (hatched column); and DPPE with ad libitum feeding (solid column). *a*, Significantly different (p <0.01) from propyleneglycol control, fed ad libitum. *b*, Significantly different (p <0.01) from fasted control.

These results show that Tam and DPPE have two important effects that differ from the effect of fasting. Although fasting and Tam and DPPE treatment reduce plasma triacylglycerols and VLDL mass, only Tam and DPPE cause a reduction in LDL and HDL phospholipid mass in comparison to the propyleneglycol control. The decrease is due largely to a selective decrease in molecular species containing palmitic or stearic acid in combination with oleic or linoleic acid, with very little change in the absolute amount of molecular species containing

arachidonic and docosahexaenoic acids. A comparable effect with fasting is achieved only after an 18-hr fast. The data are consistent with retention of long chain polyunsaturated phospholipids through selective synthetic or catabolic processes.

DISCUSSION

The mechanisms controlling serum lipoprotein phospholipid concentration and composition in humans or experimental animal models are only partially understood (1). Studies of lipoprotein and phospholipid synthesis in choline deficiency (20,22), in dietary cholesterol loading (22) or during hormonal modulation with estrogen in the chick (23–25) suggest that the synthesis of many of the lipoprotein constituents is coordinated and that phospholipid synthesis is required for release of lipoproteins. Very little is known about the regulation of molecular species composition other than an obvious dietary effect of unsaturated fatty acids (7,15). The cockerel represents a convenient model for the study of lipoprotein metabolism because the lipoprotein classes and apolipoproteins have many similarities to those of the human (26), except that exogeneous lipid absorbed in the gut is transported via the portal vein as portomicrons (27). Furthermore, the unusual lipoproteins associated with the egg-laying cycle in the female are absent, but can be induced in cockerel by the administration of estrogen (26).

The marked changes in the composition of lipoprotein phospholipids of the cockerel within 9 hr of treatment with Tam or DPPE provide an interesting model to assess the effect on lipoprotein composition of rapid changes in the molecular species composition of phospholipids. The lipid composition of lipoproteins of cockerels injected with propyleneglycol vehicle but fed ad libitum compare well with other investigations (23,26,28). The lower content of triacylglycerol in LDL and HDL in Tam and DPPE treatment compared with the propyleneglycol control is probably due to the marked decrease in plasma triacylglycerol and VLDL concentrations during treatment. Cholesterol tends to decrease or increase the order parameters for fatty acyl chains of bilayer phospholipids in the gel or the liquid crystalline state, respectively, while increasing unsaturation of the fatty acid chains tends to decrease the order parameters (29). Furthermore, a change in the amount of unsaturated fatty acids in cell membranes is balanced by an increase in the cholesterol content in order to maintain the order parameters of the fatty acyl chains (30). Although there is a rather dramatic alteration in the molecular species of PC, there is no clear trend in the ratio of cholesterol to phospholipid in the

TABLE 1

Lipoprotein Total Lipid Mass in Cockerel Serum

Treatment[a]	Lipoprotein[b] (mg/100 ml) LDL	HDL
9 Hr		
Fast (5)	86.6 ± 8.3	359.6 ± 15.7
PG (8)	89.5 ± 6.0	283.7 ± 13.7
Tam (7)	36.3 ± 7.4*,†	221.2 ± 22.5**,†
DPPE (6)	43.0 ± 4.4*,†	238.6 ± 17.5**,†
18 Hr		
Fast (5)	84.9 ± 8.8	315.6 ± 15.9
PG (8)	94.9 ± 9.0	314.5 ± 13.6
Tam (8)	53.2 ± 11.7**,††	222.1 ± 13.4*,†
DPPE (5)	81.5 ± 11.8*	294.9 ± 35.2

[a]9 Hr and 18 hr refer to the length of time between treatment and blood collection. Fast, propyleneglycol injected control fasting; PG, fed propyleneglycol-injected control; Tam, treated with tamoxifen (100 mg/kg body weight) in propyleneglycol; DPPE, treated with N-N-diethyl-2-[(4-phenylmethyl)-phenoxy]ethanamine. HCL, 100 mg/kg body weight) in propyleneglycol. The cockerels were allowed access to food and water for the propyleneglycol, Tam and DPPE treatment. Numbers in parentheses indicate numbers of birds.
[b]Results are mean ± standard deviation. LDL, low density lipoprotein; HDL, high density lipoprotein.
*Significantly different from propyleneglycol, $p<0.01$.
†Significantly different from fasting, $p<0.01$.
**Significantly different from propylene glycol, $p<0.05$.
††Significantly different from fasting, $p<0.05$.

TABLE 2

Composition of Low Density Lipoprotein in Cockerel Serum

Treatment[a]	Lipid[b] (wt %) FC	CE	PL	TAG	FC/PL
9 Hr					
Fast	7.6 ± 0.6	48.7 ± 3.6	31.5 ± 2.6	12.1 ± 3.4	.241
PG	9.3 ± 1.9	45.6 ± 4.5	27.7 ± 3.1	14.3 ± 3.6	.336
Tam	9.3 ± 4.0	53.2 ± 7.4	27.7 ± 3.1	8.1 ± 3.2	.336
DPPE	11.5 ± 1.2	50.4 ± 3.4	28.0 ± 2.1	9.5 ± 3.4	.411
18 Hr					
Fast	6.9 ± 0.5	55.2 ± 1.6	31.0 ± 2.1	7.9 ± 3.5	.220
PG	10.2 ± 2.7	47.1 ± 4.1	31.8 ± 2.2	10.8 ± 2.7	.321
Tam	12.4 ± 4.3	40.4 ± 6.4	32.5 ± 3.4	14.6 ± 10.0	.382
DPPE	11.8 ± 1.6	44.6 ± 3.7	31.8 ± 3.4	11.1 ± 2.6	.371

[a]Treatment conditions as described in Table 1.
[b]FC, free cholesterol; CE, cholesteryl ester; PL, phospholipids; TAG, triacylglycerol.

TABLE 3

Composition of High Density Lipoprotein in Cockerel Serum

Treatment[a]	Lipid[b] (wt %)				
	FC	CE	PL	TAG	FC/PL
9 Hr					
Fast	3.0 ± 0.3	45.6 ± 0.9	43.7 ± 0.8	7.6 ± 1.2	.069
PG	3.6 ± 0.6	40.2 ± 4.1	47.9 ± 3.1	8.3 ± 2.2	.077
Tam	3.8 ± 0.8	45.1 ± 4.0	46.2 ± 3.3	3.9 ± 1.2	.082
DPPE	3.9 ± 0.9	46.1 ± 1.5	46.5 ± 2.5	3.5 ± 1.4	.084
18 Hr					
Fast	2.5 ± 0.2	47.2 ± 1.9	42.2 ± 0.5	8.8 ± 1.7	.059
PG	3.8 ± 0.7	38.7 ± 3.3	51.2 ± 4.3	4.6 ± 0.9	.074
Tam	4.7 ± 0.9	40.3 ± 3.1	51.5 ± 1.6	4.2 ± 3.5	.091
DPPE	4.5 ± 0.7	39.3 ± 2.9	51.9 ± 2.7	4.3 ± 1.3	.087

[a]Treatment conditions as described in Table 1.
[b]FC, free cholesterol; CE, cholesteryl ester; PL, phospholipids; TAG, triacylglycerol.

TABLE 4

Composition of Molecular Species of Phosphatidylcholine (PC) from Serum Lipoproteins Following Treatment

Molecular species[a]	Sample time after treatment[b]							
	9 Hr (wt %)				18 Hr (wt %)			
	Fast	PG	Tam	DPPE	Fast	PG	Tam	DPPE
HDL								
C34	1.8	0.7	0.3	0.1		0.3	2.1	0.2
C36	20.4	30.0	21.5	21.0	15.8	26.5	22.2	27.6
C38	31.6	38.4	30.5	29.6	27.0	35.5	29.9	29.6
C40	35.8	23.8	34.9	36.1	48.5	29.8	36.4	31.7
C42	10.4	7.1	12.9	13.2	8.7	7.8	10.3	10.8
LDL								
C34	4.5	3.1	3.7	6.9	5.8	3.9	6.5	4.6
C36	25.2	36.3	32.9	27.0	21.9	30.3	31.3	36.3
C38	32.1	36.7	34.7	28.1	27.3	33.3	30.0	25.1
C40	30.2	23.8	24.8	33.3	39.0	28.4	24.7	25.6
C42	8.0	0.5	3.7	4.8	5.9	3.9	6.5	8.5

[a]HDL, high density lipoprotein; LDL, low density lipoprotein. Molecular species of diacylglycerols of PC are identified on the basis of their retention time equivalent to triacylglycerols. Each carbon number species of diacylglycerol contains a total acyl carbon number that is 2 less than the present designation (i.e., C34 contains a total of 32 acyl carbon atoms.)

[b]Conditions and abbreviations are as given in Table 1. Results are from the analyses of pooled lipoproteins from 5–8 birds in each treatment group. Estimates of absolute amounts for individual birds are given in Figures 3–6.

lipoproteins. While fasting and Tam and DPPE treatment all increase the relative amount of polyunsaturated fatty acid in the PC, fasting samples have the lowest ratio of cholesterol to phospholipid, while Tam and DPPE treatment have the highest values. Thus it is unclear whether the increased fluidity imparted tothe lipoproteins by the increase in the polyunsaturated fatty acids is balanced by an increase in the content of unesterified cholesterol.

Although the mechanism for the modulation of the molecular species of PC in this model is not clear from these studies, a number of possibilities may be considered. As discussed elsewhere (Lazier, C.B., and Breckenridge, W.C., submitted for publication), Tam competes with estrogen for the estrogen receptor. Estrogen action is very low in the cockerel. Futhermore, DPPE does not compete for the estrogen receptor (16), but induces the same effect on lipoproteins as Tam. Thus it is probable that the influence of both compounds on lipoproteins may not be via the estrogen receptor and inhibition of the estrogen effect on lipoprotein synthesis, but by some other mechanism, possibly involving other high affinity sites (antiestrogen binding sites) in the liver, or other sites such as protein kinase C, which

TABLE 5

Fatty Acid Composition of Phosphatidylcholine

	Sample time after treatment[b]							
	9 Hr (wt %)				18 Hr (wt %)			
Fatty acid[a]	Fast	PG	Tam	DPPE	Fast	PG	Tam	DPPE
LDL				N.D.			N.D.	
16:0	24.7	25.3	26.5		19.8	22.1		25.6
16:1	0.5	0.8	0.4		0.9	0.7		0.3
18:0	29.3	27.5	30.5		29.0	29.4		23.3
18:1	12.6	16.3	14.1		12.0	16.3		14.9
18:2	11.9	15.7	7.7		10.2	16.6		12.8
20:3	0.6	1.0	1.7		0.7	0.3		0.6
20:4	11.9	6.7	10.3		14.2	11.3		11.2
20:5	0.6	0.5	0.3		1.0	0.2		0.4
22:5	5.2	2.6	1.3		5.2	3.1		2.8
22:6	5.3	3.4	7.5		6.9	6.2		7.9
HDL								
16:0	21.7	26.1	23.2	23.5	22.3	23.9	26.0	26.3
16:1	0.8	0.6	0.5	0.1	0.3	0.1	0.1	0.2
18:0	18.0	25.8	26.5	25.3	27.9	25.0	23.2	27.6
18:1	18.2	17.3	12.3	11.0	11.1	12.0	10.0	9.2
18:2	18.9	16.5	10.7	10.7	10.3	15.3	12.0	12.4
20:3	0.1	1.1	2.1	0.7	0.1	1.9	1.4	0.4
20:4	12.2	7.1	15.2	16.6	16.4	11.7	14.8	15.0
20:5	0.8	0.4	0.8	1.7	1.0	1.6	1.1	1.5
22:5	1.3	2.0	1.7	1.0	2.6	0.9	1.1	1.2
22:6	8.6	2.7	6.9	9.5	7.8	7.6	10.2	11.2

[a]LDL, low density lipoprotein; HDL, high density lipoprotein. Fatty acid identified by number of acyl carbon atoms and double bonds.

[b]Treatment as described in Table 1. N.D., not done—sample lost.

is inhibited by Tam with a Ki in the micromolar range (31).

Tam inhibits the estrogen-induced synthesis of VLDL apo B (32), but has no effect on general protein synthesis in cockerel liver. The low levels of LDL and HDL during treatment may be due to decreased formation of LDL and HDL through lipolysis of VLDL. It is unlikely that an alteration in molecular species of HDL phospholipids that is associated with an overall decrease in lipoprotein mass would be due to a selective removal of lipoproteins of specific phospholipid composition. A general reduction in the synthesis of HDL precursor phospholipids is a more plausible explanation. Reports of direct synthesis of HDL precursor lipids and apolipoproteins by chick liver (33) have also shown that the HDL in the Golgi are largely spherical structures and have many similarities to plasma HDL. Recent investigations (1) in cultured rat hepatocytes indicate that PC is synthesized by the CDP-choline diacylglycerol pathway as well as by methylation of PE, which is formed by CDP-ethanolamine diacylglycerol pathway or from decarboxylation of phosphatidylserine. However, PC that is destined for lipoproteins appears to arise primarily from pools of phospholipids synthesized by the CDP-choline pathway and from methylation of PE that is derived from decarboxylation of phosphatidylserine. Early in vivo data for phospholipid metabolism suggested (34) that rat liver and plasma PC, which contained linoleic acid, were formed by the CDP-choline pathway, whereas PC-containing polyunsaturated fatty acids were formed via methylation of PE. Further studies of phospholipid biosynthesis in this model will be required to resolve whether Tam and DPPE inhibit selectively certain pathways of phospholipid biosynthesis in order to produce the alteration in molecular species.

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Effect of Cholestyramine on Bile Acid Metabolism in Conventional Rats

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Effects of cholestyramine on biliary secretion of cholesterol, phospholipids and bile acids and fecal excretion of sterols and bile acids were examined in Wistar male rats. Six rats were fed a basal diet, and the other six were fed a basal diet supplemented with 5% cholestyramine for eight days. Bile flow and biliary secretion of bile acids and phospholipids (per hour per rat) decreased with cholestyramine treatment, while biliary cholesterol secretion (per hour per rat) remained unchanged. In the biliary bile acid composition, a marked increase of chenodeoxycholic acid with a concomitant decrease of β-muricholic acid was observed in cholestyramine-treated rats. Fecal excretion of total sterols and bile acids increased about three- and four-fold, respectively, after cholestyramine treatment. The increase of fecal bile acids derived from cholic acid was more predominant than that derived from chenodeoxylcholic acid, resulting in an increase of the cholic acid group/chenodeoxycholic acid group ratio.

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Cholestyramine is a nonabsorbable bile acid–binding anion exchange resin, and its administration brings about interruption of the enterohepatic circulation of bile acids (1). Clinically, cholestyramine is widely used as a hypocholesterolemic agent in man (2,3).

Gustafsson et al. (4) reported that cholestyramine treatment stimulated HMG CoA reductase and cholesterol 7α-hydroxylase, the rate-limiting enzymes in cholesterol and bile acid synthesis, in germ-free rats. They also examined fecal bile acid compositions and found that cholic acid synthesis was stimulated to a greater extent than chenodeoxycholic acid synthesis. In contrast to the results in the germ-free rats, Huff et al. reported that the fecal excretion of cholic acid was unchanged, whereas that of dihydroxy bile acids were increased several times by cholestyramine feeding in conventional rats (5).

It has been reported that biliary secretion of bile acids and phospholipids decreased while biliary cholesterol secretion remained unaltered by cholestyramine treatment in baboons and hamsters (6,7). In guinea pigs, cholestyramine treatment has been shown to lower the ratio of bile acid to cholesterol in the bile and to induce gallstones (8). However, the effects of cholestyramine on biliary lipid secretion in rats are controversial (9,10). Turley et al. reported that cholestyramine treatment had no effect on biliary lipid secretion (9). On the other hand, Innis reported that biliary bile acid secretion increased, but that biliary secretion of cholesterol and phospholipids was unchanged (10).

To clarify the effect of cholestyramine on bile acid metabolism in conventional rats, we analyzed biliary lipid secretion and fecal excretion of sterols and bile acids, with special reference to bile acid compositions of bile and feces in conventional rats fed cholestyramine diets. The results indicated that biliary secretion of bile acids and phospholipids decreased, while biliary cholesterol secretion was unchanged, and that cholic acid synthesis increased to a greater extent than did chenodeoxycholic acid synthesis in cholestyramine-treated conventional rats. In addition, we discuss the conversion of chenodeoxycholic acid to β-muricholic acid during the enterohepatic circulation on the basis of the changes of the bile acid compositions induced by cholestyramine treatment.

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MATERIALS AND METHODS

Animals. Male Wistar rats weighing about 220 g were used. They were maintained in an air-conditioned room (25 ± 1 C, 50–60% humidity) with free access to water. Six control rats were fed a basal diet (Type MF, Oriental Yeast Co., Tokyo, Japan), and the other six were fed a basal diet supplemented with 5% cholestyramine. The composition of the basal diet was as follows: 24.0% protein, 5.1% lipids, 54.5% carbohydrate, 3.2% fiber, 6.2% ash and 7.0% water. The content of cholesterol was 0.09%. Rats were individually caged and given the diet for eight days. Feces were collected for two days before rats were killed. On the ninth day rats were anesthetized by intraperitoneal sodium pentobarbital injection (50 mg/kg), and the bile duct was cannulated with PE-10 polyethylene tubing (Clay Adams, Franklin Lakes, New Jersey) to collect bile for 2 hr.

Biliary lipid determination. Biliary bile acids, cholesterol and phospholipids were determined as reported previously (11–13). Bile was extracted with ethanol; one volume of bile was poured into 20 vol of ethanol, boiled about 5 min and filtered after cooling down to room temperature. An aliquot of the filtrate was evaporated to dryness under a stream of nitrogen, and the residue was hydrolyzed in 3 ml of 1.25 N NaOH for 6 hr at 120 C. Cholesterol was extracted with diethyl ether; bile acids were then extracted with diethyl ether after acidification with 2 N HCl. Cholesterol was determined by gas liquid chromatography (GLC) on a 1% SE-30 column. Bile acids were converted to methyl ester trifluoroacetate derivatives and determined by GLC using 1.5% QF-1 and 1.5% AN-600 columns. Phospholipids were determined by the method of Gomori (14). Lithogenic index was calculated by the formula of Thomas and Hofmann (15).

Fecal sterol and bile acid determination. Fecal sterols and bile acids were determined as described previously with a slight modification (11–13). Feces were homogenized in water; an aliquot was extracted with 15 ml of ethanol at 90 C for 1 hr three times and with 5 ml of petroleum ether twice at room temperature. Extracts were filtered, combined and evaporated to dryness under reduced pressure. The residue was hydrolyzed in 4 ml of 1.25 N NaOH at 120 C for 6 hr. After extraction of sterols

with 3 vol of diethyl ether three times, the hydrolysate was acidified to pH 1–2 with 2 N HCl and bile acids were extracted with 3 vol of diethyl ether three times. The sterols and bile acids were quantified by GLC. Absorption efficiency was calculated as follows: 100 × (1-daily fecal bile acid excretion/daily biliary bile acid secretion) (16).

Statistical analysis. The results are expressed as mean ± SEM. Student's t-test was used to determine statistical significance.

RESULTS

Table 1 shows the effects of cholestyramine on body weight, bile flow, biliary secretion of cholesterol, phospholipids and bile acids and the lithogenic index. Body weight gains were not different between the two groups. The bile flow and biliary secretion of phospholipids and bile acids decreased in the cholestyramine-fed rats when they were expressed in terms of ml or mg per hr per rat. The concentration of bile acids decreased, but those of cholesterol and phospholipids remained unchanged. The lithogenic index increased in the treated rats. Biliary bile acid compositions are compared in Table 2. The biliary bile acids in the control rats consisted of cholic acid (50.2%), β-muricholic acid (17.8%), chenodeoxycholic acid (6.5%), hyodeoxycholic acid (6.3%), ursodeoxychoic acid (5.8%), deoxycholic acid (4.9%), α-muricholic acid (2.2%) and small amounts of the other bile acids. In the cholestyramine-fed rats, chenodeoxycholic acid markedly increased (25.9%), β-muricholic acid decreased (5.3%), α-muricholic acid increased (5.7%) and hydeoxycholic acid almost disappeared. The ratio of cholic acid and its related bile acids to chenodeoxycholic acid and its related bile acids (CA group/CDCA group ratio) remained unchanged.

The fecal excretion of sterols increased about three-fold (Table 3) and that of bile acids about four-fold (Table 4) after cholestyramine treatment. ω-Muricholic acid (31.2%), hyodeoxycholic acid (21.1%), deoxycholic acid (17.6%) and lithocholic acid (9.6%) were the major components in the fecal bile acids of the control rats. Cholestyramine treatment increased deoxycholic acid (46.0%) and lithocholic acid (29.9%) and decreased ω-muricholic acid (3.9%) and hydeoxycholic acid (2.4%), resulting in a marked increase of CA group/CDCA group ratio. The absorption efficiency (the ratio of daily intestinal bile acid absorption to daily biliary bile acid secretion) was reduced to a great extent in cholestyramine-treated rats.

DISCUSSION

Cholestyramine had no effect on biliary lipid composition in obese subjects or patients with gallstones and showed lithogenic effect in normal subjects, while it decreased cholesterol saturation in patients with primary hyperlipoproteinemia (17–20). In rats, Turley et al. (9,21) reported no changes in bile flow or biliary lipid secretion in the rats fed 2% cholestyramine diets for two weeks. Innis (10) reported that biliary bile acid secretion was increased in the rats fed 3% cholestyramine diets for about three weeks, but bile flow and biliary secretion of cholesterol and phospholipids were unchanged. These studies were done with female Sprague-Dawley rats. In contrast, our present study with male Wistar rats showed that bile flow and biliary secretion of bile acids and phospholipids decreased but biliary cholesterol secretion remained unchanged when rats were fed 5% cholestyramine diets for eight days. Similar results have been reported in baboons and hamsters (6,7). These conflicting results in rats might be due to the difference in sex or in dose and duration of cholestyramine treatment.

In the present study, the fecal excretion of bile acids and neutral sterols increased significantly by cholestyramine treatment, which is in agreement with other reports (4,5,22). The increase of the CA group/CDCA

TABLE 1

Effects of Cholestyramine on Bile Flow, Biliary Cholesterol, Phospholipids and Bile Acids and Lithogenic Index in Rats

	Control (n = 6)	Cholestyramine (n = 6)
Initial body weight (g)	225.5 ± 4.5[a]	220.8 ± 5.8
Final body weight (g)	285.5 ± 4.6	272.2 ± 10.0
Bile flow (ml/hr)	1.00 ± 0.12	0.68 ± 0.04[b]
Cholesterol (mg/hr/rat)	0.141 ± 0.013	0.126 ± 0.029
Phospholipids (mg/hr/rat)	1.52 ± 0.27	0.80 ± 0.15[b]
Bile acids (mg/hr/rat)	7.29 ± 0.96	2.51 ± 0.23[b]
Cholesterol (mg/ml)	0.144 ± 0.007	0.188 ± 0.047
Phospholipids (mg/ml)	1.51 ± 0.19	1.17 ± 0.20
Bile acids (mg/ml)	7.40 ± 0.72	3.67 ± 0.27[b]
Lithogenic index	0.229 ± 0.013	0.459 ± 0.081[b]

[a]Mean ± SE.
[b]Statistically significant compared to control value ($p < 0.05$).

TABLE 2

Effects of Cholestyramine on Biliary Bile Acid Composition in Rats

	Control (n = 6)	Cholestyramine (n = 6)
Cholic acid group (%)	55.5 ± 1.4[a]	54.5 ± 3.8
Cholic acid	50.2 ± 1.5	52.3 ± 3.7
Deoxycholic acid	4.9 ± 1.0	1.8 ± 0.2[b]
3α, 12α-Dihydroxy-7-oxo-	0.4 ± 0.03	0.4 ± 0.1
Chenodeoxycholic acid group	41.7 ± 1.4	43.0 ± 3.9
Chenodeoxycholic acid	6.5 ± 0.5	25.9 ± 2.9[b]
Ursodeoxycholic acid	5.8 ± 0.2	4.5 ± 0.9
α-Muricholic acid	2.2 ± 0.1	5.7 ± 0.8[b]
β-Muricholic acid	17.8 ± 0.8	5.3 ± 0.7[b]
Hyodeoxycholic acid	6.3 ± 0.7	n.d.[c]
3α-Hydroxy-6-oxo-	1.6 ± 0.2	0.2 ± 0.04[b]
3α-Hydroxy-7-oxo-	1.1 ± 0.03	0.8 ± 0.1
Lithocholic acid	0.4 ± 0.1	0.6 ± 0.1
Others[d]	2.8 ± 0.3	2.5 ± 0.3
CA group/CDCA group ratio	1.34 ± 0.08	1.40 ± 0.25

[a]Mean ± SE.
[b]Statistically significant compared to control value ($p < 0.05$).
[c]Not detectable.
[d]Others comprise unidentified peaks with relative retention times of 0.74 and 0.85.

group ratio in feces of the cholestyramine-fed rats is compatible with the result in germ-free rats (4). In man, synthesis of cholic acid also increased to a greater extent than that of chenodeoxycholic acid by cholestyramine treatment (23). Provided that bile acid metabolism of rats fed cholestyramine is in a steady state after eight days, the increase of the CA group/CDCA group ratio implies much more increase of cholic acid synthesis than chenodeoxycholic acid synthesis. These results agree with the concept that cholic acid is formed from newly synthesized cholesterol to a greater extent than chenodeoxycholic acid (11,24,25). The difference of binding affinity of various bile acids to cholestyramine might not be responsible for the increase of the CA group/CDCA group ratio in feces because cholestyramine has been reported to bind taurocholic acid less effectively than taurochenodeoxy cholic acid (26).

There was no difference between the CA group/CDCA group ratios in the bile of control and cholestyramine-treated rats. As previously reported (13), the ratio in the bile is higher than that in feces in untreated rats, probably due to more efficient active transport of cholic acid than of chenodeoxycholic acid in the lower ileum (27). Absorption efficiencies in control and cholestyramine-treated rats were 95% and 38%, respectively (Table 4). The CA group/CDCA group ratio in the bile was similar to that in the feces of cholestyramine-treated rats (1.40 vs 1.05), while the ratios in control rats were much different (1.34 vs 0.39). These data suggest that absorption of bile acids, especially cholic acid, was prevented by cholestyramine. The biliary excretion of bile acids in the rats with biliary drainage for eight days was around 2 mg/hr (unpublished data); the value was similar to that found in the present experiments with cholestyramine given for eight days, suggesting that a major part of biliary bile acids was composed of newly synthesized bile acids in the cholestyramine-treated rats.

Chenodeoxycholic acid markedly increased and β-muricholic acid decreased in the biliary bile acid composition after cholestyramine treatment. This result in conventional rats corresponds well to the increase of chenodeoxycholic acid in the feces in cholestyramine-

TABLE 3

Effects of Cholestyramine on Fecal Excretion of Sterols in Rats

	Control (mg/day per rat; n = 6)	Cholestyramine (mg/day per rat; n = 6)
Total sterols	14.11 ± 1.12[a]	40.26 ± 3.12[b]
Coprostanol	7.37 ± 0.46	25.11 ± 1.70[b]
Cholesterol	6.74 ± 0.95	15.15 ± 1.76[b]

[a]Mean ± SE.
[b]Statistically significant compared to control value ($p < 0.05$).

TABLE 4

Effects of Cholestyramine on Fecal Excretion of Bile Acids in Rats

	Control (mg/day per rat; n = 6)	Cholestyramine (mg/day per rat; n = 6)
Total bile acids	8.83 ± 0.34[a]	38.26 ± 3.46[b]
Cholic acid group	2.38 ± 0.15 (27.1)	17.94 ± 1.52[b] (47.3)
Deoxycholic acid	1.55 ± 0.13 (17.6)	17.42 ± 1.56[b] (46.0)
Cholic acid	0.42 ± 0.06 (4.7)	0.09 ± 0.06[b] (0.2)
3α, 12α-Dihydroxy-7-oxo-	0.29 ± 0.06 (3.4)	0.17 ± 0.08 (0.4)
7β-Cholic acid	0.12 ± 0.01 (1.4)	0.27 ± 0.16 (0.7)
Chenodeoxycholic acid group	6.31 ± 0.35 (71.3)	18.10 ± 2.04[b] (46.9)
Lithocholic acid	0.84 ± 0.04 (9.6)	11.58 ± 1.37[b] (29.9)
Hyodeoxycholic acid	1.86 ± 0.34 (21.1)	0.91 ± 0.32 (2.4)
Chenodeoxycholic acid	< 0.01	0.94 ± 0.23[b] (2.4)
Ursodeoxycholic acid	< 0.01	0.70 ± 0.15[b] (1.9)
α-Muricholic acid	0.25 ± 0.02 (2.8)	1.14 ± 0.17[b] (3.0)
β-Muricholic acid	0.19 ± 0.04 (2.2)	0.96 ± 0.14[b] (2.4)
ω-Muricholic acid	2.79 ± 0.46 (31.2)	1.48 ± 0.23[b] (3.9)
3α-Hydroxy-6-oxo-	0.28 ± 0.03 (3.2)	0.29 ± 0.18 (0.8)
3α-Hydroxy-7-oxo-	0.10 ± 0.04 (1.2)	0.08 ± 0.04 (0.2)
Others[c]	0.14 ± 0.01 (1.6)	2.22 ± 0.28[b] (5.8)
CA group/CDCA group ratio	0.39 ± 0.04	1.05 ± 0.13[b]
Absorption efficiency (%)	94.5 ± 0.1	37.6 ± 8.0[b]

[a]Mean ± SE. Values in parentheses represent % of the total bile acids.
[b]Statistically significant compared to control value ($p < 0.05$).
[c]Others comprise unidentified peaks with relative retention times of 0.74 and 0.85.

treated germ-free rats (4). Chenodeoxycholic acid has been reported to be transformed into β-muricholic acid through α-muricholic acid in bile fistula rats (28,29) and isolated hepatocytes (30,31). The first step of this conversion is 6β-hydroxylation of chenodeoxycholic acid. An in vitro study on the 6β-hydroxylase system using microsomes obtained from cholestyramine-treated and untreated rats showed that cholestyramine treatment exerted little influence on the activity (32,33). As mentioned above, a major part of biliary bile acids was newly synthesized bile acids in cholestyramine-treated rats, so it seems likely that newly synthesized chenodeoxycholic acid is not utilized as a substrate of chenodeoxycholic acid 6β-hydroxylase before its first secretion into bile. Botham and Boyd (30) demonstrated that when rats were totally biliary drained for 44 hr, chenodeoxycholic acid content of the bile showed a rapid fall in the first 10 hr and then a rise beginning after 24 hr, whereas β-muricholic acid content of the bile also fell rapidly during the first 10 hr but showed no significant rise afterward. These results in bile fistula rats agree with our results in cholestyramine-treated rats.

In the biliary bile acid composition of the present experiments, α-muricholic acid increased in addition to the marked increase of chenodeoxycholic acid and the decrease of β-muricholic acid by interruption of enterohepatic circulation of bile acids due to cholestyramine treatment. α-Muricholic acid has been reported to be converted to β-muricholic acid through a 7-keto intermediate (34,35). Therefore, it is conceivable that β-muricholic acid is formed from chenodeoxycholic acid and even from α-muricholic acid during the enterohepatic circulation in normal rats.

In conclusion, biliary secretion of bile acids and phopholipids decreased, while that of cholesterol remained unchanged in conventional rats fed cholestyramine diets. Cholestyramine treatment also brought about an increase of fecal excretion of sterols and bile acids and an increase of CA group/CDCA group ratio in the fecal bile acid composition, which implies much more increase of cholic acid synthesis than chenodeoxycholic acid synthesis. There was a marked increase of chenodeoxycholic acid with a concomitant decrease of β-muricholic acid in the bile of cholestyramine-treated rats, suggesting the formation of β-muricholic acid from chenodeoxycholic acid during the enterohepatic circulation.

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Effect of Dietary Lipids on the Lipid Composition and Phospholipid Deacylating Enzyme Activities of Rat Heart[1]

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Rats were fed lard-enriched (17%) or corn oil–enriched (17%) diets and were compared with rats fed a low fat (4.5%) diet. Cardiac protein, DNA, phospholipid (PL) and fatty acid (FA) compositions were analyzed. Neutral phospholipase A, lysophospholipase and creatine kinase activities in the membrane and cytosolic compartments were also investigated.

No significant modification of cardiac protein, DNA nor PL was observed among the three groups. Some alterations appeared in the FA composition. A lard-enriched diet induced a significant increase of 22:5n-3 and 22:6n-3 in heart phosphatidylcholine (PC) and phosphatidylethanolamine (PE), whereas a linoleic acid–rich diet induced a specific increase of 22:4n-6 and 22:5n-6 in these two major PL.

Compared to rats fed the low fat diet, membrane-associated phospholipase A activity, measured by endogenous hydrolysis of membrane PC and PE, showed a significant increase (+45%) for both PL in rats fed corn oil. However, the activity of membrane-associated phospholipases, measured with exogenous [1-^{14}C]dioleoyl PC, was not different among the three groups of rats. Cytoplasmic activity was decreased in rats fed corn oil, and lysophospholipase and creatine phosphate kinase activities were not significantly affected by diet.

FA modification of the long chain n-6 FA induced by corn oil may be responsible for the observed increase in phospholipase activity. Physiological implications are suggested in terms of membrane degradation and prostaglandin production.
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Cellular membranes act not only as a biological barrier but also as a finely tuned system that controls ion permeability, membrane-associated enzymes, receptors and eicosanoid production. Any alteration of membrane lipid composition may lead to cellular metabolic disorders. In normally functioning membranes, phospholipid (PL) as well as fatty acid (FA) composition are perfectly regulated. However, it is possible by dietary manipulations to alter the membrane FA composition. The heart is a tissue responsive to lipid modification of the diet. Polyunsaturated oils have a beneficial effect on the cardiovascular system (1–3). This had led several researchers to study the effect of polyunsaturated fat of either vegetable (4–8) or fish (9–11) origin on heart lipid composition. It was observed that a diet enriched in polyunsaturated fatty acids (PUFA) (18:2n-6, 18:3n-3) did not affect the PL composition of cardiac membranes or the polyunsaturated/saturated (P/S) ratio of FA. However, significant alterations were observed in PUFA of the n-3 and n-6 series. In general, a diet enriched in 18:2n-6 induced an elevation of the n-6/n-3 ratio, whereas a diet enriched in 18:3n-3 had the opposite effect (4–8). A significant decrease of this ratio was also observed when rats were fed menhaden or tuna oils, which contain substantial amounts of n-3 PUFA (10,11).

These modifications can have physiological consequences. It is known that membrane-bound enzyme activity can be influenced by the nature of lipids in the proximal micro-environment of the protein (12,13). This is probably related to the degree of unsaturation of FA, which determines, in part, the membrane fluidity and allows the motion of the enzyme in the lipid bilayer (13). Mammal studies have shown that dietary lipid manipulation could modify the activities of some membrane-associated enzymes, such as cation-transporting ATPase (13).

Another important group of membrane-bound enzymes is phospholipase A. Its activity has been described in rat (14–17) and hamster (18) hearts in the membranous systems of the cell (14–17). In addition, a cytosolic phospholipase A (19) and C (20) have been partially characterized in heart. Attention is focused on these enzymes because it is thought that in ischemic heart the observed increase of membrane PL degradation (21,22) could result from activation of intracellular cardiac phospholipases

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TABLE 1

Composition of Experimental Diets (g/100g)

	Low fat	Lard	Corn oil
Casein[a]	27.3	27.3	27.3
Lard[a]	2.2	14.8	—
Corn oil[b]	2.2	2.2	17.0
Starch[a]	34.0	26.9	26.9
Glucose[a]	23.5	18.0	18.0
Minerals[c]	5.0	5.0	5.0
Vitamins[d]	1.0	1.0	1.0
Cellulose[a]	4.8	4.8	4.8

[a]From Unité Alimentation Reationnelle (UAR) (Villemoisson, France).

[b]From CPC Europe Consumer Products (Heilbronn, Federal Republic of Germany).

[c]From UAR. Mineral mixture (stated in the Kg mixture): $CaHPO^4$, 430; KCl, 100; NaCl, 100; $MgSO^4$, 50; $FeSO^4$ $7H_2$ O, 5; Fe_2O_3, 3; $MnSO_4$ H_2O, 2.45; $CuSO_4$ $5H_2O$, 0.5; $CoSO_4$ $7H_2O$, 4×10^{-3}; $ZnSO_4$ $7H_2O$, 2; stabilized KI, 8×10^{-3} and NaF, 0.25.

[d]From UAR. Vitamin mixture (stated in Kg mixture except as noted): retinol, 1980 IU; calciferol, 600 IU; thiamin, 2; riboflavin, 1.5; vitamin B3, 3.5; pyridoxine, 1; carnitine, 15; cyanocobalamin, 5 mg; ascorbic acid, 80; alpha-tocopherol, 17; menadione, 4; nicotinic acid, 10; choline, 136; pteroylmonoglutamic acid, 0.5 mg; *p*-aminobenzoic acid, 5; and biotin, 0.03.

(23,24). This event, associated with an increase of lysoPL, could further induce an excess of calcium in the cytosol, leading to electrophysiological disorders (25). Almost no information is available on the regulation of these activities in heart. The natural substrate of cellular phospholipase A is essentially membrane PL, and any chemical modification of these lipids may affect their activities. For example, it has been shown in vitro that phospholipase A activity of a homogenate of hamster heart is considerably increased by the level of unsaturation of FA esterified in the *sn*-2 position (18). This was the case also for the erythrocyte phospholipase A (26). Our purpose in this work was to study the effect of a PUFA-rich or PUFA-poor diet on the phospholipase A activities in rat heart in relation to PL and FA composition.

MATERIALS AND METHODS

Animals, diets and feeding procedures. Three groups of 15 male Wistar rats (IFFA-CREDO, L'Arbresle, France) weighing 190–210 g were used. Each group was divided into five cages of three rats. One group was fed a low fat diet containing 4.5% (w/w) fat consisting of a lard and corn oil mixture, giving a P/S ratio of 1.2. A second group received a lard diet (14.8%, w/w) enriched with corn oil (2.2%, w/w), which served as a source of essential FA; the P/S ratio was 0.4. The third group was fed corn oil (17%, w/w); the P/S ratio was 5. The composition of the diets and their FA compositions are given in Tables 1 and 2, respectively. The rats were fed ad libitum for 8 wk and had free access to water.

Heart tissue treatment. After the feeding period, the rats were weighed and guillotined, and their hearts were excised quickly. The hearts were extensively rinsed at 4 C in Tris HCl buffer, 20 mM (pH 7.5), containing 0.22 M mannitol and 70 mM sucrose. Extraventricular tissues and vessels were removed. Hearts were weighed and pooled by three with respect to the cage distribution. They were finely minced; one-third was reserved for homogenization and the rest was subjected to lipid extraction.

The homogenization was performed at 4 C in mannitol buffer (10%, w/w) using a polytron (Kinematica-PT 10) for 2 sec at a rheostat setting of 6. The homogenate was centrifuged at 100,000 × g for 1 hr at 4 C to separate all the membrane fractions (M) from the cytosolic fraction. All fractions were immediately stored at −60 C. The lipids were extracted from fresh tissue at 4 C according to the method of Tam et al. (18). Lipids were extracted in the presence of 0.01 N HCl to minimize loss of lysoPL and 0.05% butylated hydroxytoluene as an antioxidant. Lipids were stored at −20 C under nitrogen until high performance liquid chromatographic (HPLC) and gas liquid chromatographic (GLC) analyses.

HPLC and GLC. Choline and ethanolamine containing PL were separated by HPLC using a Waters-Associate liquid chromatographic system (Milford, Massachusetts). PL were separated as previously described (27). The analysis was performed on an NH_2-Lichrosorb 10 μm (Merck, Darmstadt, Federal Republic of Germany using a mixture of n-hexane, isopropanol, methanol and water as solvent system. The elution order was phosphatidylcholine (PC), sphingomyelin (SPH), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE); the separation was achieved in 45 min. PL were quantified by phosphorus determination (28).

TABLE 2

Fatty Acid Composition of Diets

Fatty acid	Low fat	Lard	Corn oil
16:0	20.24	26.7	11.6
16:1	0.27	2.37	—
18:0	8.04	12.5	0.18
18:1n-9	35.09	41.3	26.8
18:2n-6	33.58	14.8	60.0
18:3n-3	0.73	0.66	0.85
20:0	0.29	0.21	0.41
20:1	0.46	0.8	—
20:2	0.20	0.3	—
Saturated	28.57	39.4	12.2
Unsaturated	70.33	60.2	87.6
P/S[a]	1.21	0.40	5.0

[a]Polyunsaturated to saturated fatty acid ratio.

Fatty acid methyl esters (FAME) of PC and PE were prepared by 5% sulfuric acid in methanol at 50 C under N_2 for 3 hr. FAME were extracted twice with n-hexane and analyzed by a gas liquid chromatograph (Girdel 3000, Paris, France) equipped with a peak integrator (Delsi, Enica 10, Suresne, France) and a 50-m capillary column (Spirawax FS 1493, Spiral, Dijon, France). Injector and detector temperatures were set at 220 C, and the oven was temperature programmed (5 min at 180 C, 1 C/min to 205 C and 25 min at 205 C). FAME were identified by comparison to retention time of standards (Interchim, Paris, France).

Phospholipase and lysophospholipase assays. Phospholipase activity on endogenous PL was assayed as recently described (29). Briefly, the membrane fraction (M) (800 μl), obtained from ultracentrifugation at 100,000 × g, was incubated at 37 C at pH 8.4 with 8 mM calcium for 1 hr. Reaction was stopped by adding chloroform/methanol (2:1, v/v), and lipids were extracted according to the method of Folch et al. (30). All extraction steps were performed at 4 C to avoid chemical autolysis of PL. The extracts were submitted to HPLC analysis. Activity was calculated by measuring the decrease of lipid phosphorus in the PC and PE. The contribution of thermal degradation of endogenous PL in measurement of phospholipase A activity was considered. Extracted cardiac PL were resuspended in the buffer and dispersed by sonication. They were incubated at 37 C for 1 hr, extracted and separated on HPLC as described above. Compared with a sample at 4 C, no significant thermal degradation of PC and PE was observed, indicating that no nonenzymic hydrolysis occurred during the phospholipase assay.

Due to the very low concentration of PL in the cytoplasm, labeled exogenous substrate was needed to measure the cytoplasmic phospholipase A activity. Membrane-bound phospholipase A activity was also measured using labeled exogenous PC. The assay with exogenous PC was performed as previously described (17) at pH 8.4 using [1-^{14}C]dioleoylPC (N.E.N., Paris, France) with a specific activity of 6.35 Bq/nmol. The substrate was dis-

persed by sonication in the form of unilamellar liposomes. All the enzymatic measurements were linear with respect to protein concentration and time. A sample without protein served as a blank.

Lysophospholipase was assayed at pH 8.0 as previously described (17) using [1-^{14}C]lysopalmitoylPC (N.E.N.) (14.7 Bq/nmol LPC). Creatine phosphate kinase activity after activation with N-acetyl L-cystein was performed as described by Szasz et al. (31). Assays were measured using an automatic Multistat III (Instrumentation Laboratory, Spokane, Washington) at 30 C.

Miscellaneous. Cholesterol was determined with the cholesterol esterase–cholesterol oxidase kit (Boehringer Mannheim GmbH, Mannheim, FRG) using a two-point kinetic method (32). Assays were performed with an automatic Multistat III at 30 C. Proteins were determined according to the method of Lowry et al. (33). Desoxyribonucleic acid content was measured as described by Volkin and Cohn (34).

DioleoylPC, lysopalmitoylPC and all PL standards were obtained from Sigma (99% pure) (Coger, Paris, France). Solvents were of HPLC grade and were filtered through 0.2 μm Millipore filter before use.

Statistical analysis. Results presented in the tables are means ± SEM of five groups of three pooled rat hearts. Statistical significance of mean differences between dietary groups was investigated by analysis of variance.

RESULTS

Over the 8-wk feeding period, there was no significant difference ($p>0.05$) in body (402 ± 11 g) and heart (1.1 ± 0.1 g) weights between rats on the low fat diet and those on fat-enriched diets.

Table 3 shows the effect of diets on the protein/DNA ratio, total lipid phosphorus and cholesterol content. No significant variation was observed in any of these parameters among the three groups of rats.

The PC and PE compositions are shown in Table 3. Neither the lard-enriched nor the corn oil–enriched diet produced changes in PL composition, which agrees with previous studies performed with other types of fat (7,8,10). The FA compositions of PC and PE are given in Table 4. The saturated FA were resistant to dietary manipulation both in PC and PE inasmuch as the percentages of palmitic and stearic acid were not significantly altered by the diets. The same observation goes for the P/S ratio, which confirms recent data obtained with rats fed other vegetable oils (4,6,7).

Significant alterations in the PUFA, however, were induced by the diets. In the PC, linoleic acid (18:2n-6) was significantly reduced in rats fed the linoleic acid–poor diet (lard). By contrast, the metabolic products of this acid, formed by elongation and desaturation (20:4n-6, 22:4n-6 and 22:5n-6), were not significantly modified by the lard-rich diet. This supports the idea that, in PC, linoleic acid is efficiently converted into C20 and C22 n-6 PUFA, to maintain their concentrations in the normal cell. The same suggestion can be made for PE, where a 2.4-fold reduction of 18:2n-6 (compared to the low fat group) was observed with the lard-enriched diet. Except for a slight decrease of the 20:4n-6 (20%), the C22 n-6 species were maintained at levels comparable to those of the low fat diet. In the same way, a linoleic acid–rich diet (corn oil) produced a significant increase of the C22 n-6 PUFA in PC and PE, which supports an efficient conversion of linoleic acid into C22 n-6 PUFA. Feeding a lard-enriched diet resulted in a dramatic increase of the PUFA n-3 family in both PC and PE. In the PC, this increase is about threefold compared to the low fat group and twofold in the PE.

Phospholipase, lysophospholipase and creatine phosphate kinase activities were measured in the total cardiac membrane fraction (Table 5) and in the cytoplasm. The endogenous hydrolysis of membrane PL showed that PE was better hydrolyzed than PC independent of the diet given. The specific activity, expressed per mg of DNA, was about 30% higher for PE compared to PC in rats fed low fat and corn oil diets, and 70% higher in rats fed the lard diets. When comparing the specific activity of the phospholipase A among the three diet groups, the corn oil–enriched diet induced a significant increase in phospholipase A activity compared to that of the low fat diet. The hydrolysis of PC was about 48% higher in rats fed corn oil compared to that of the low fat diet. Similarly, the hydrolysis of PE was increased by 42% compared to

TABLE 3

Protein, DNA, Cholesterol, PC and PE Composition of Hearts from Rats fed Various Diets

	Low fat	Lard	Corn oil
mg Protein/mg DNA	55.4 ± 2.4	67.3 ± 2.6	57.0 ± 4.9
μmol Lipid phosphorus/g of heart	22.2 ± 0.9	21.6 ± 1.4	22.6 ± 1.3
μmol Cholesterol/g of heart	1.73 ± 0.25	2.2 ± 0.25	2.32 ± 0.18
PC	52.2 ± 0.9	53.0 ± 0.6	52.8 ± 1.3
LPC	1.05 ± 0.06	1.08 ± 0.02	1.09 ± 0.05
PE	41.9 ± 1.0	41.0 ± 0.6	41.5 ± 1.2
LPE	1.16 ± 0.39	1.04 ± 0.12	0.96 ± 0.21
Sph	3.7 ± 0.1	3.9 ± 0.04	3.7 ± 0.14

PC, phosphatidylcholine; LPC, lysophosphatidylcholine; PE, phosphatidylethanolamine; LPE, lysophosphatidylethanolamine; Sph, sphingomyelin. Values are means ± SEM; n = 5 for each group. Each n represents the pool of three rat hearts. Phospholipid values are expressed as molar percentage.

TABLE 4

Fatty Acid Composition of Phosphatidylcholine and Phosphatidylethanolamine of Heart from Rats Fed Various Diets

	Phosphatidylcholine			Phosphatidylethanolamine		
Fatty acid	Low fat	Lard	Corn oil	Low fat	Lard	Corn oil
16:0	19.1 ± 1.8	15.0 ± 0.4	17.3 ± 1.0	8.0 ± 0.4	8.3 ± 0.4	7.5 ± 0.4
18:0	34.9 ± 3.1	34.9 ± 0.5	34.3 ± 0.8	29.4 ± 0.8	30.2 ± 1.2	30.0 ± 0.9
18:1n-9	5.1 ± 0.32^{a**}	3.7 ± 0.22^{b**}	3.5 ± 0.12^{b**}	4.6 ± 0.2 ^{a*}	3.9 ± 0.2 ^{b*}	4.3 ± 0.15^{a*}
18:2n-6	8.0 ± 1.6 ^{a*}	3.6 ± 0.12^{b*}	8.6 ± 0.4 ^{a*}	4.5 ± 0.4 ^{a*}	1.9 ± 0.05^{b*}	6.6 ± 0.4 ^{c*}
20:0	0.26 ± 0.04	0.1 ± 0.03	0.25 ± 0.04	0.33 ± 0.05	0.12 ± 0.05	0.22 ± 0.02
20:1	0.15 ± 0.03	<0.1	0.1 ± 0.03	<0.1	<0.1	<0.1
20:2	<0.1	<0.1	0.3 ± 0.04	<0.1	<0.1	0.28 ± 0.02
20:4n-6	28.9 ± 3.5	36.1 ± 0.7	31.0 ± 1.4	29.7 ± 0.6 ^{a*}	24.0 ± 0.9 ^{b*}	22.4 ± 0.8 ^{b*}
22:4n-6	0.62 ± 0.06^{a**}	0.8 ± 0.03^{a**}	1.26 ± 0.1 ^{b**}	2.6 ± 1.3 ^{a**}	2.4 ± 0.12^{a**}	4.1 ± 0.2 ^{b**}
22:5n-6	0.63 ± 0.07^{a**}	0.64 ± 0.06^{a**}	1.14 ± 0.14^{b**}	6.2 ± 0.6 ^{a**}	4.6 ± 0.4 ^{a**}	10.0 ± 0.3 ^{b**}
22:5n-3	0.4 ± 0.05^{a**}	1.3 ± 0.05^{b**}	0.47 ± 0.02^{a**}	1.3 ± 0.01^{a**}	2.9 ± 0.1 ^{b**}	1.27 ± 0.05^{a**}
22:6n-3	1.3 ± 0.18^{a**}	3.3 ± 0.08^{b**}	1.6 ± 0.1 ^{a**}	12.9 ± 0.8 ^{a**}	22.6 ± 1.0 ^{b**}	12.9 ± 0.4 ^{a**}
Saturated	54.2 ± 4.9	49.9 ± 0.8	51.8 ± 1.8	37.7 ± 0.9	38.6 ± 1.5	37.7 ± 1.0
Monounsaturated	5.2 ± 0.2 ^{a*}	3.7 ± 0.2 ^{b*}	3.6 ± 0.1 ^{b*}	4.7 ± 0.2 ^{a*}	3.94 ± 0.2 ^{b*}	
4.4 ± 0.1 ^{ba*}						
Polyunsaturated	39.8 ± 0.7	45.8 ± 0.7	44.3 ± 1.9	57.3 ± 1.0	58.4 ± 2.2	57.5 ± 1.0
n-6	38.1 ± 5.5	41.1 ± 0.8	42.0 ± 2.1	43.0 ± 0.7 ^{a**}	32.9 ± 1.7 ^{b**}	42.3 ± 1.4 ^{a**}
n-3	1.65 ± 0.3 ^{a**}	4.62 ± 0.07^{b**}	2.07 ± 0.01^{a**}	14.2 ± 0.9 ^{a**}	25.44 ± 1.2 ^{b*}	14.15 ± 0.4 ^{a*}

Values are means ± SEM; n = 5 for each group, and each n represents a pool of three rat hearts. Differences in horizontal means in a class of phospholipids without a common superscript are statistically significant (*, p <0.05; **, p <0.01). If no superscript appear in a row, the differences of the mans are not statistically significant (p>0.05).

that of the low fat diet. Consequently, the sum of the hydrolysis of PC and PE was increased by 45%, and the total activity (nmol PL hydrolyzed/hr/g heart) was significantly increased (P<0.01) by 58% (3,054 in rats fed corn oil vs 1,931 in rats fed low fat diet). In general, rats fed lard diets tended toward a slight increase of enzyme activity, but this increase was not significant (p>0.05) compared to rats fed a low fat diet. The total activity per g of heart was 2,226 nmol PL hydrolyzed/hr in the group fed the lard diet and was significantly different (p<0.01) from the corn oil group.

The phospholipase activity of the membrane fraction using exogenous [1-^{14}C]dioleoylPC is given in Table 5. For all three diets, the measured specific activity was seven- to ninefold lower than that obtained with the endogenous method, confirming recent data obtained with rat heart homogenates (29). However, with the exogenous substrate, no significant diet modifications of phospholipase specific activity were observed. Therefore, in this kind of study, the use of endogenous substrate allowed observation of changes in enzyme activity induced by diet.

The phospholipase activity in the cytoplasm was investigated in the three groups of animals. Compared to the low fat group, a significant decrease of 35% in the specific activity (per mg of DNA) was induced by the corn oil diet, while the decrease in total activity (per g of heart) was 30%. The lard diet did not alter significantly cytoplasmic phospholipase A activity. Lysophospholipase activity was investigated both in the total membrane fraction (Table 5) and in the cytoplasm. Lard and corn oil diets did not significantly modify the membrane-bound lysophospholipase specific activity nor the cytoplasmic lysophospholipase activity.

Creatine phosphate kinase was chosen as an associated-membrane enzyme not involved in lipid metabolism. Although its activity tended to increase with high fat diets (Table 5), it was not significant (p>0.05).

DISCUSSION

Diet-induced changes in FA composition of heart PL have been investigated recently, and the data show that oils of vegetable or fish origin induce alterations in the proportions of the n-3 and n-6 families in PUFA (4–10). Such modifications in heart lipids might be responsible for some alterations in membrane function, such as mitochondrial ATPase activity (35) or sarcolemma-bound enzyme activity (36). The purpose of this study was to investigate the possible influence of two different kinds of fat, lard and corn oil, on lipolytic cardiac enzymes involved in PL catabolism.

Both lard- and corn oil–enriched diets induced no modification in the content of protein, DNA, total PL and cholesterol. In addition, the distribution between the PC and PE remained unchanged. This agrees with previous data obtained with other edible oils (4,6,7,10) and emphasized the ability of heart tissue to regulate its protein and lipid membrane composition even under diet lipid manipulation.

However, diet-induced FA modifications were evident in the proportion of PUFA in the PC and PE. A linoleic acid–poor diet (lard) induced a marked increase in the n-3 PUFA family (22:5n-3 and 22:6n-3) both in PC and PE, probably because the FA desaturase system has a better affinity for n-3 than n-6 FA (37). Consequently, in the presence of a lard-enriched diet, the n-6/n-3 ratio was reduced by a factor of more than 2 both in PC and PE.

TABLE 5

Membrane-Associated Phospholipase A, Lysophospholipase and Creatine Phosphate Kinase Activities in Hearts from Rats fed Various Diets

	Phospholipase (endogenous method)			Phospholipase, exogenous method nmol FA/hr/mg DNA	Lysophospholipase, nmol FA/hr/mg DNA	Creatine phosphate kinase units/mg DNA
	nmol PC/hr/ mg DNA	nmol PE/hr/ mg DNA	nmol PE + PC/hr mg DNA			
Low fat	356.7 ± 54.0[a*]	483.7 ± 33.0[a*]	840.4 ± 65.1[a**]	120.5 ± 9.1	915.0 ± 42.0	5857 ± 558
Lard	365.8 ± 14.0[a*]	634.4 ± 30.5[ab]	1000.0 ± 32.6[a*]	138.8 ± 6.5	1155.1 ± 61.9	7052 ± 310
Corn oil	529.9 ± 47.5[b*]	686.6 ± 70.0[b*]	1215.0 ± 73.0[b*]	133.6 ± 8.5	1081.9 ± 101.0	7072 ± 637

Values are means ± SEM; n = 5 for each group (except n = 4 for lysophospholipase assay in the control), and each n represents the pool of three rat hearts. Symbols of significance are identical to those of Table 4. Phospholipase activity is expressed as nmol of phospholipid hydrolyzed (endogenous method) and as nmol of [1-^{14}C]oleic acid liberated (exogenous method). Lysophospholipase is expressed as nmol of [1-^{14}C]oleic acid liberated. For creatine kinase one unit corresponds to a μmol of creatine phosphate hydrolyzed/min.

This decrease has also been obtained with rats fed sheep kidney fat (6,7) or low erucic acid rapeseed oil (4), which are rich in either palmitic or oleic acid, respectively, like lard.

A decrease in linoleic acid in PC and PE could also be explained by the fact that the levels of 22:4n-6 and 22:5n-6 (metabolic products of 18:2n-6) were maintained at levels comparable to that of the control. In the same way, rats fed a linoleic acid–rich diet showed significantly increased levels of 22:4n-6 and 22:5n-6, associated in PE with a slight decrease of 20:4n-6. Other authors also have reported high increases of 22:4n-6 and 22:5n-6 (4,38) and almost no change in 20:4n-6 (4,7,38) in rats fed linoleic acid–rich oil. These data suggest that 22:4n-6 and 22:5n-6 could have some physiological function in the heart, especially in PE, which would justify maintaining these high levels.

Experimental evidence suggests that membrane FA alteration in various tissues might modify the activity of some membrane-bound enzymes (13). We have investigated this possibility with cardiac phospholipases and lysophospholipases. It is interesting to note that in each group of rat hearts, PE is better hydrolyzed than PC. This could be explained by the higher content of PUFA in PE and the preference of cardiac phospholipases for greater unsaturation (18). This would be supported by the general observation that corn oil induced a significant rise in phospholipase activity in the membrane-bound fraction for both PC (+48%) and PE (+42%). This rise in activity does not appear to be the result of an increase in enzyme synthesis since the activity, measured on exogenous [1-^{14}C]dioleoyl PC, was not increased in rats fed corn oil. Interestingly, the use of endogenous membrane PL as substrate, permitted measurement of increased phospholipase activity. This might indicate that the enzyme was more active, because corn oil induced some specific alterations in the physicochemical properties of membrane PL.

In rats fed corn oil, the observed increase of 22:4n-6 and 22:5n-6 in both PC and PE and the increase of 18:2n-6 in PE may influence the phospholipase activity in two manners. First, it is known that small changes in the unsaturation of membrane PL may produce a modification in the enzyme conformation or motion, inducing in some cases a higher Vmax. This phenomenon has been observed for heart membrane-bound enzymes (36,37) and some other enzymes (12,13), such as the microsomal acyl CoA:cholesterol acyltransferase in liver (39). In this way, phospholipase A–susceptible domains have been proposed in platelet membranes (40,41). These domains would be preferentially attacked by phospholipase A and could be created by modification of FA unsaturation. Second, membrane PL are also the substrate of membrane-bound phospholipases. Since their unsaturation may influence heart phospholipase activity (17,18), it is possible that the diet-induced changes provide a better enzyme activity on the substrate.

In conclusion, we suggest that specific alterations induced by corn oil in some FA of the n-6 family lead to an increase of membrane-associated phospholipase activity. At this stage of investigation, it is impossible to predict if this increase of activity might be responsible for some membrane-associated electrophysiological disorders. Indeed, even if lysoPL are produced in higher proportions, lysophospholipases are probably sufficiently active to rapidly remove these compounds (17). This is confirmed by the fact that lysoPC and lysoPE contents of heart did not change under the various diet conditions.

On the other hand, the observed increase of phospholipase A activity in rats fed corn oil may have physiological consequences in heart prostaglandin production. The content of arachidonic acid in heart PL was not increased even when rats were fed a linoleic acid–rich diet. Such a result can be achieved by an increased conversion to longer chain fatty acids, as discussed above, and a greater liberation of arachidonic acid, the precursor of prostaglandins, by a more active phospholipase. The results presented here should be considered in light of other studies showing that manipulation of dietary lipids affects heart prostaglandin synthesis (42–44), in particular prostacyclin (PGI_2) production, which increases in a linoleic acid–rich diet (43,44). The rise in PGI_2 may have a beneficial effect on heart function (45–47).

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Effects on Plasma Lipids and Fatty Acid Composition of Very Low Fat Diets Enriched with Fish Or Kangaroo Meat

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The effects of very low fat diets (<7% energy) enriched with different sources of long chain (C20 and C22) polyunsaturated fatty acids (PUFA) on plasma lipid levels and plasma fatty acid composition were studied in 13 healthy volunteers. Three diets provided 500 g/day of tropical Australian fish (rich in arachidonic acid and docosahexaenoic acid), southern Australian fish (rich in docosahexaenoic acid) or kangaroo meat (rich in linoleic and arachidonic acids). The fourth diet was vegetarian, similarly low in fat but containing no 20- and 22-carbon PUFA. Subjects ate their normal or usual diets on weeks 1 and 4 and the very low fat diets in weeks 2 and 3. Weighed food intake records were kept, and weeks 2, 3 and 4 were designed to be isoenergetic with week 1.

Plasma cholesterol levels fell significantly on all diets within one week. There were reductions in both low density (LDL) and high density lipoprotein (HDL) cholesterol levels, with effects on HDL cholesterol being more consistent. There were no consistent or significant effects on total triglyceride levels despite the high carbohydrate content of the diets. On all diets the percentage of linoleic acid fell in the plasma phospholipid and cholesteryl ester fractions, while the percentage of palmitic acid in the phospholipids and cholesteryl esters and palmitoleic acid in the cholesteryl ester fraction rose on all diets. The percentage of arachidonic acid rose in the phospholipid and cholesteryl esters on the two diets that were good sources of this fatty acid (tropical fish and kangaroo meat). The percentage of docosahexaenoic acid also rose on the two diets that were the richest sources of this fatty acid (the fish diets), and the percentage of eicosapentaenoic acid rose in the phospholipid and cholesteryl esters in proportion to the dietary level of this fatty acid (southern fish > kangaroo > tropical fish). The changes in fatty acid composition were almost completely reversed within seven days of returning to the usual higher fat diets.
Lipids 22, 523–529 (1987).

We have previously shown that two types of diets traditionally eaten by aborigines from the northern coastal regions of Australia are associated with a threefold increase in the proportion of arachidonic acid in plasma lipids (1,2). The first was a diet derived almost exclusively from tropical seafood (1), which we have subsequently analyzed and found to be a rich source of arachidonic acid (1,3). The second diet was one in which kangaroo meat and freshwater fish (linoleic and arachidonic acid-rich) were the major sources of fat (2). Both diets were very low in fat despite being rich in fish and red meat (2,4). Plasma triglycerides fell on both diets, and bleeding time increased (1,2). Plasma cholesterol levels, which were not high initially, were not significantly affected by the diets, even when they were rich in lean red meat.

The aim of the present study was to follow up these original observations by examining under controlled conditions the effects on plasma lipoprotein lipids and plasma fatty acid composition of four diets similarly low in fat but containing quite different polyunsaturated fatty acids (PUFA): three contained either 500 g/day of tropical fish (rich in arachidonic and docosahexaenoic acids), southern Australian fish (rich in docosahexaenoic acid) or kangaroo meat (rich in linoleic and arachidonic acids). The fourth diet was vegetarian, with a similarly low fat content and polyunsaturated/saturated ratio but containing no long chain (>20 carbon) PUFA.

METHODS

Thirteen healthy, weight-stable subjects (seven women and six men) participated in these studies. They had a mean age of 31.3 ± 2.8 years and a mean body mass index of 21.2 ± 0.5 kg/m^2. Each subject participated in 1–4 diet studies with a break of at least three months between diets. The protocol was approved by the Ethics Committee of the Royal Melbourne Hospital, Victoria, Australia.

Diets. The four experimental diets were designed to meet two objectives, namely, to provide a minimum of fat (<7% of total energy intake) and to be isoenergetic such that no loss of body weight occurred over the 2-wk period. An experienced research dietitian instructed each subject individually to eat a wide variety of suitable foods to ensure the nutritional adequacy and maximize the palatability of each diet. Subjects were familiar with all food items apart from the fish and kangaroo meat that were supplied. An extensive list of recipes was provided with each diet. Allowable foods included skim milk and other nonfat dairy products. Grains and cereal products such as bread and pasta were encouraged. All vegetables and fruits except avocado and olives were allowed. Legumes, except soybeans, were also encouraged. Sugars, jams, soft drinks and candies were used as a source of energy. Fats, oils, nuts, meat and fish (other than those supplied) as well as commercial foods with added fat were excluded from the diet. Due to the high bulk and low energy density of the experimental diets, subjects with a high basal energy requirement (>2500 kcal/day) found it difficult to maintain their energy intake. In an attempt to overcome this problem, high energy supplementary drinks (high carbohydrate, no fat, 250 kcal each) were recommended. Subjects were advised to have one drink after breakfast and another before retiring at night. Every subject was provided with a set of kitchen scales and standard food-record sheets. All food and beverages consumed over each 2-wk diet period plus the pre- and

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Abbreviations: PUFA, polyunsaturated fatty acids; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein.

TABLE 1

Sample 2000-kcal Low Fat Menus

3 Sample breakfasts (common to all four diets)

300 g oatmeal porridge 100 ml skim milk 2 tsp sugar 2 medium peaches 1 slice wholemeal toast 10 g marmalade jam	200 ml skim milk 200 g baked beans (homemade) 2 slices wholemeal toast	200 ml orange juice 60 g very low fat muesli (homemade) 150 ml skim milk 2 slices wholemeal toast 15 g nonfat cottage cheese 10 g yeast extract

Tropical fish	Southern fish	Kangaroo	Vegetarian
Midday meal			
250 g Barramundi, poached with green peppercorns, capers and Dijon mustard 150 g tossed salad (lettuce, onion, tomato, cucumber, radishes, peppers, large pear)	250 g Rock Ling, poached in mango sauce with dry white wine and nonfat yogurt 150 g baked potato 70 g pumpkin, steamed 60 g spinach, steamed	250 g Kangaroo in curry sauce (no oil) 100 g nonfat yogurt 200 g steamed rice ½ cantaloupe	4 slices wholemeal bread 2 tsp prepared mustard 80 g salad vegetables (tomato, mung beans, grated carrot, beetroot) 1 medium banana
Evening meal			
250 g Barramundi, chow-mein style (1 T. sherry, 1 T. soy sauce) 150 g mixed vegetables 200 g rice noodles	250 g Nanagai, grilled with lemon juice 250 ml split pea soup 1 wholemeal bread roll tossed salad (oil-free dressing)	250 g kangaroo shasliks 100 g steamed potato 70 g broccoli 60 g carrot 200 g fruit salad 100 g nonfat yogurt	1 bowl minestrone soup (no oil) 2 cups cooked spaghetti 180 g tomato and vegetable sauce 150 g tossed salad, no dressing
Snacks (in addition to above meals three snacks were eaten each day)			
(e.g.) 2 slices of bread or 1 crumpet with jam or honey	small banana plus 200 ml nonfat yogurt	400 ml carbonated soft drink or 250 ml high energy supplement drink	2 Rye Vita crackers with 20 g nonfat cottage cheese 30 g tomato plus 40 g dried apricots

postcontrol weeks were recorded. Typical 2000-kcal menus for each diets are presented in Table 1. The breakfasts and most snacks were interchangeable. Dietary analyses were performed using the Microdiet Computer Software package based on the British Food Tables (5). The fat content of the tropical fish, southern fish and kangaroo meat was determined by analysis in our laboratory (3,6).

The daily intake of saturated and monounsaturated fatty acids and linoleic acid were calculated using the Microdiet data base. The levels of the 20- and 22-carbon PUFA were calculated from our own data of the total lipid content and fatty acid composition of a variety of foods.

Experimental protocol. Each diet study ran for four weeks. Fasting blood samples were taken before the study began and at weekly intervals after for the measurement of plasma lipoprotein lipid and fatty acid compositions. During the first week the subjects remained on their usual diets, weighing and recording food intake. They began the experimental diets during the second and third weeks and returned to their usual diets for the fourth week. Weighed food-intake records were maintained throughout the four weeks. Body weights were also monitored regularly.

Lipoprotein lipid analysis. Concentrations of cholesterol and triglyceride in fasting plasma were measured enzymatically after enzymatic hydrolysis on a Cobas-B10 Centrifugal Analyser using commercially available kits (Cholesterol Enzymatic Merckotest, E. Merck, Darmstadt, FRG; Triglyceride Rapid Test, Roche, Basle, Switzerland). The normal range for cholesterol concentrations in fasting plasma is 3.5–6.0 mmol/l and for triglycerides is 0.5–2.0 mmol/l. Very low density lipoproteins (VLDL) were separated by 16-hr ultracentrifugation of plasma at 40,000 rev/min in a Beckman L-50 Ultracentrifuge. High density lipoproteins (HDL) were separated within 2 hr of blood collection from other plasma lipoproteins that had been precipitated by heparin-manganese chloride. Lipids in low density lipoproteins (LDL) were calculated from the difference between whole plasma and VLDL and HDL (7). Cholesterol concentrations were determined for all three lipoprotein fractions. Triglyceride concentrations were measured in whole plasma and VLDL only.

Plasma fatty acid analysis. Fasting blood samples were collected in heparinized tubes, and the lipids were extracted from the plasma by chloroform/methanol extraction (8). Internal standards of cholesterol heptadecanoate (Nu-Chek-Prep, Elysian, MN) and di-hepta-decanoyl-phosphatidylcholine (Sigma Chemical Co., St. Louis, MO) were added to the plasma samples prior to lipid extrac-

tion. The lipid extracts were separated by thin layer chromatography (1,2), and the fatty acid methyl esters of the cholesteryl ester and phospholipid fractions were formed as described previously (9). The fatty acid methyl esters were separated using an 8 m × 0.22 mm ID fused silica bonded phase (BP-20) capillary column (SGE, Melbourne, Australia). The gas liquid chromatograph was equipped with flame ionization detectors and was programmed from 100 to 190 C at 8 C/min with a helium carrier gas flow rate of 50 cm/sec. Standard methyl esters (Nu-Chek-Prep) were routinely chromatographed to determine the identity of the fatty acid methyl esters and to determine the detector response to the different esters. Quantitative response factors were determined (using Nu-Chek-Prep standards) and were used in the calculation of the data. The capillary column provided adequate separation of the following critical methyl esters: 18:3n-3 from 20:0 and 20:1; 20:5n-3 from 22:0 and 22:1; 20:4n-6 from 20:3n-6 and 20:3n-3.

Statistical analyses. The paired t-test was used to compare results within a study, and the unpaired t-test to compare results between studies. All results are expressed as mean ± SEM; significance was taken as $p < 0.05$.

TABLE 2

Dietary Composition Before, During and After the Four Experimental Diets (Mean ± SEM)

Diet	Energy (KJ)	% of total energy				Dietary fiber (g)	Cholesterol (mg)	P/S ratio[a]
		Protein	Carbohydrate	Fat	Ethanol			
Baseline	9188±292	14.7±0.4	43.5±0.8	36.3±0.9	5.5±0.5	24.7±1.4	277±13	0.45±0.04
Southern fish (10)	7871±373***	27.9±1.3***	59.2±1.1***	5.9±0.4***	7.0±0.9	31.7±3.8**	266±4	1.78±0.06***
Tropical fish (11)	7512±431**	29.9±1.3***	56.3±1.7***	7.2±0.6***	6.6±1.0	28.4±2.2	263±5	1.18±0.07***
Kangaroo meat (10)	8651±545	28.7±1.2***	57.8±2.2***	6.9±0.4***	6.6±1.0	34.6±3.5***	299±4	1.33±0.08***
Vegetarian (7)	7553±885**	15.0±0.6***	71.3±1.9***	6.6±0.6	7.1±1.2	43.4±4.9**	13±2***	1.42±0.18***
Postdiet	9632±301*	14.5±0.4	44.5±0.9	35.7±1.0	5.3±0.5	23.4±1.1	325±22*	0.42±0.03

Paired t-test comparing baseline diet 1 with the other five diets. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.
[a]P/S: polyunsaturated/saturated.

TABLE 3

Cholesterol Concentration in Fasting Whole Plasma and in Lipoprotein Fractions During the Dietary Studies (mmol/l, mean ± SEM)

Diet	Baseline (day 0)	Diet (day 7)	Diet (day 14)	Post diet (day 21)
Total plasma cholesterol				
Southern fish (10)	4.54±0.22	3.83±0.12***	3.51±0.12***	4.20±0.12
Tropical fish (11)	4.75±0.27	4.08±0.19*	3.86±0.22*	4.13±0.16
Kangaroo (10)	5.22±0.43	4.42±0.41**	4.17±0.42**	4.86±0.37
Vegetarian (7)	4.54±0.32	3.59±0.25	3.44±0.27**	4.12±0.36
VLDL cholesterol				
Southern fish (10)	0.12±0.02	0.09±0.05	0.11±0.04	0.10±0.03
Tropical fish (11)	0.17±0.03	0.18±0.03	0.14±0.03	0.17±0.03
Kangaroo (8)	0.23±0.05	0.31±0.09	0.27±0.09	0.22±0.06
Vegetarian (7)	0.16±0.03	0.21±0.05	0.17±0.03	0.13±0.05
LDL cholesterol				
Southern fish (10)	3.17±0.21	2.79±0.12***	2.50±0.15***	3.00±0.13
Tropical fish (8)	3.13±0.24	2.75±0.14	2.57±0.22	2.60±0.14*
Kangaroo (8)	3.63±0.51	3.07±0.39*	3.05±0.43**	3.22±0.25
Vegetarian (7)	2.91±0.28	2.29±0.22	2.38±0.26	2.88±0.34
HDL cholesterol				
Southern fish (10)	1.26±0.13	0.98±0.10***	0.93±0.08**	1.16±0.08
Tropical fish (8)	1.53±0.14	1.20±0.14**	1.21±0.19*	1.42±0.13
Kangaroo (8)	1.48±0.09	1.21±0.08*	1.19±0.06*	1.58±0.20
Vegetarian (7)	1.35±0.13	1.08±0.13**	0.89±0.12***	1.11±0.10

Statistically significant differences from baseline day 0: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

TABLE 4

Ratio of Cholesterol in Low Density to High Density Lipoprotein Lipid Fractions During the Dietary Studies (Mean ± SEM)

Diet	Day 0	Day 7	Day 14	Day 21
Southern fish (10)	2.78 ± 0.32	3.19 ± 0.45	2.96 ± 0.49	2.75 ± 0.31
Tropical fish (8)	2.21 ± 0.21	2.40 ± 0.17	2.22 ± 0.34	1.96 ± 0.19
Kangaroo (8)	2.38 ± 0.25	2.55 ± 0.30	2.54 ± 0.26	2.15 ± 0.18
Vegetarian (7)	2.35 ± 0.29	2.39 ± 0.41	3.09 ± 0.71	2.73 ± 0.44

RESULTS

The changes in dietary composition during the dietary studies are shown in Table 2. Although there was a trend for energy intake to fall on all four diets, it was not statistically significant. However, on all diets except the kangaroo diet, energy intake increased significantly when the baseline diet was resumed, although there were no significant differences between the two baseline periods (weeks 1 and 4). The marked fall in proportion of energy derived from fat on all diets was compensated for by increased carbohydrate intake in the vegetarian diet and increased protein and carbohydrate intakes on the other three diets containing lean meat or fish. Cholesterol intake was extremely low on the vegetarian diet, but unchanged on the other three diets relative to the baseline periods. Dietary fiber increased on all diets, the effect being most marked with the vegetarian diet. The P/S ratio increased from 0.4 on the baseline diet to over 1 on the vegetarian, tropical fish and kangaroo diets and to almost 2 on the southern fish diet.

Fasting cholesterol fell 19–24% over the 2-wk period, with the most pronounced fall occurring in the first week of the diet (Table 3). The effect of the diets on cholesterol levels appeared to be equally rapidly reversible on all diets, and levels had risen significantly within one week of resuming the baseline diet for all diets except tropical fish. The fall in total cholesterol on these diets was due to reductions in both HDL and LDL cholesterol, with effects on HDL cholesterol being more consistent. The HDL-cholesterol levels fell significantly on all four diets and rose significantly within one week of resuming the baseline diet. LDL-cholesterol levels behaved in a similar fashion for all diets except tropical fish, where the fall on the diet was less pronounced (and not statistically significant) and more attenuated in that it remained low one week after resuming the baseline diet. The ratios of LDL/HDL cholesterol over the study period are shown in Table 4. Although there was a trend to higher ratios during all four diets, there were no statistically significant effects.

Triglyceride levels in fasting plasma and VLDL were not affected by the diets (Table 5). There were trends toward higher triglyceride levels on all diets; however, they were not statistically significant. The exception was on the tropical fish diet, where there was a significant increase in triglyceride levels after one week that disappeared by two weeks.

The fatty acid composition of the foods used in supplementing the low fat diets is given in Table 6. Kangaroo meat and tropical and southern Australian fish are all low in fat with a high proportion of PUFA. Kangaroo meat contains predominantly n-6 PUFA, with linoleic and arachidonic acids being the most abundant. However, it is important to note that kangaroo meat contains significant amounts of n-3 PUFA (linolenic acid and its more polyunsaturated derivatives). Tropical fish are rich in both the n-6 and n-3 PUFA, with arachidonic and docosahexaenoic acids being the most abundant. Southern Australian fish, in contrast, contain predominantly the n-3 PUFA, with docosahexaenoic acid being the most abundant.

TABLE 5

Triglyceride Concentrations in Fasting Total Plasma and VLDL During the Dietary Studies (mmol/l, mean ± SEM)

Diet	Baseline (day 0)	Diet (day 7)	Diet (day 14)	Postdiet (day 21)
Total plasma triglycerides				
Southern fish (10)	0.72 ± 0.04	0.71 ± 0.06	0.76 ± 0.04	0.62 ± 0.05
Tropical fish (11)	0.77 ± 0.10	0.89 ± 0.07*	0.84 ± 0.05	0.74 ± 0.05
Kangaroo (10)	0.85 ± 0.10	1.21 ± 0.24	1.16 ± 0.24	0.96 ± 0.13
Vegetarian (7)	0.75 ± 0.10	0.92 ± 0.10	0.94 ± 0.13	0.81 ± 0.13
VLDL triglycerides				
Southern fish (10)	0.16 ± 0.02	0.14 ± 0.05	0.16 ± 0.07	0.14 ± 0.02
Tropical fish (10)	0.25 ± 0.04	0.31 ± 0.04	0.20 ± 0.03	0.25 ± 0.04
Kangaroo (10)	0.31 ± 0.07	0.52 ± 0.17	0.46 ± 0.16	0.43 ± 0.09
Vegetarian (7)	0.24 ± 0.06	0.32 ± 0.05	0.33 ± 0.06	0.27 ± 0.05

* $p < 0.05$ (paired t-test comparing baseline diet with the other diets).

The estimated daily dietary intake of saturated, monounsaturated and the different PUFA is shown in Table 7. In the baseline periods, the intake of saturated, monounsaturated and linoleic acids was 31, 30 and 13 g/day, respectively. In all experimental periods, the levels of each of these three groups of fatty acids declined to about 2–3 g/day. The baseline diet contained 20- and 22-carbon PUFA of both the n-6 and n-3 types with arachidonic and docosahexaenoic being the most significant. The vegetarian diet contained no 20- and 22-carbon PUFA, whereas the other three low fat diets were particularly enriched in the long chain PUFA derived from the main foods characteristic of each diet; southern fish with eicosapentaenoic and docosahexaenoic acids, tropical fish with arachidonic and docosahexaenoic acids and kangaroo meat with arachidonic, eicosapentaenoic and docosapentaenoic acids.

The changes in fatty acid composition of plasma phospholipids and cholesteryl esters after two weeks on the four experimental diets is presented in Table 8. Some changes appeared to be in response to the low fat diet per se, since they occurred in all four diets, while other changes (particularly those pertaining to the long chain PUFA) were quite diet-specific. In both phospholipids and cholesteryl esters, the proportion of linoleic acid fell, while that of palmitic and palmitoleic acid rose during all diets and returned to baseline values within one week of resuming the normal diet. The changes observed in the C20 and C22 PUFA reflected the fatty acid composition of the major dietary components; the proportion of arachidonic acid increased on the two diets that were good sources of this PUFA (tropical fish and kangaroo meat); the proportion of docosahexaenoic acid rose in both plasma lipid fractions on the two diets that were good sources of this PUFA (southern fish and tropical fish); and eicosapentaenoic acid increased most markedly on the southern fish diet. The quantitatively largest changes in fatty acid composition were the fall in linoleic acid and the increases in arachidonic and docosahexaenoic acids. The fatty acid changes from day 0 to day 14 were almost completely reversed within 7 days after subjects resumed their normal diets.

DISCUSSION

In this study of the effects on lipoprotein lipids and fatty acid composition in fasting plasma during four very low fat diets enriched with different long chain PUFA, some effects (consistently observed on all four diets) appeared to be simply attributable to the low dietary fat, while others (specific to the different diets) were attributable to differences in long chain PUFA composition.

The changes in lipoprotein composition in response to all four diets were similar. There was a rapid reduction in total cholesterol within one week and a slower return toward baseline values one week after resuming the normal diet. The fall in total cholesterol was due to falls in both LDL- and HDL-cholesterol levels, with the effects being more consistent on HDL cholesterol. Indeed, there was a trend to increased LDL/HDL cholesterol ratios on all four diets, although it was not statistically significant. These results are consistent with the observations of

TABLE 6

Fatty Acid Composition of the Major Sources of Long Chain Polyunsaturated Fatty Acids in the Low Fat Diets (% Total Fatty Acids of the Fresh, Raw Food)

	Kangaroo meat	Tropical fish	Southern Australian fish
14:0	1[a]	2[b]	2[c]
16:0	15	22	18
16:1	2	4	3
18:0	12	9	6
18:1	22	15	13
18:2n-6	17	5	1
18:3n-3	5	1	—
20:1	—	—	2
20:3n-6	1	1	1
20:4n-6	7	11	4
20:5n-3	2	2	7
22:4n-6	—	2	1
22:5n-6	—	3	1
22:5n-3	2	2	2
22:6n-3	1	10	31
Total lipid	1.4%	1.0%	0.7%

[a]Other components include 16- and 18-carbon aldehydes, 17:0, 17:1, 20:2, 22:4.

[b]Other components include 16- and 18-carbon aldehydes, 15:0, 17:0, 20:1, 20:2, 22:0.

[c]Other components include 15:0, 17:0, 17:1, 18:3, 18:4, 20:2.

TABLE 7

Estimated Fatty Acid Intakes (g/day) of Baseline and Low Fat Diets

			Polyunsaturated				
Diet	Saturated	Monounsaturated	18:2n-6	18:3n-3	20:4n-6	20:5n-3	22:6n-3
Baseline diet	30.7[a]	29.5	11.42	0.86	0.07[b]	0.04	0.09
Southern fish	2.1	2.4	2.64	0.24	0.14	0.25	1.08
Tropical fish	2.5	2.3	2.40	0.26	0.56	0.11	0.51
Kangaroo meat	3.2	3.7	3.55	0.58	0.49	0.14	0.07
Vegetarian	2.2	2.4	3.14	0.37	0	0	0

[a]Values from the baseline and postdiet periods (weeks 1 and 4).

[b]Values for the 20- and 22-carbon polyunsaturated fatty acids calculated from total lipid content and fatty acid composition of foods used in this study.

TABLE 8

Fatty Acid Composition of Plasma Phospholipids and Cholesteryl Esters Before, During and After Dietary Studies (Percentage of Total Fatty Acids, Mean ± SEM, Number of Subjects in Parentheses)

	Study day[a]	16:0	16:1	18:0	18:1	18:2n-6	20:3n-6	20:4n-6	20:5n-3	22:5n-3	22:6n-3
Phospholipids											
Southern fish (10)	0	28.2 ± 1.0	0.9 ± 0.1	12.6 ± 0.6	12.2 ± 0.5	25.7 ± 1.0	2.8 ± 0.2	10.8 ± 0.8	1.0 ± 0.1	0.9 ± 0.1	4.0 ± 0.3
	14	32.5 ± 1.4**	1.3 ± 0.2	10.8 ± 0.6**	11.0 ± 0.3*	16.1 ± 0.7***	2.3 ± 0.2	12.0 ± 0.7	2.8 ± 0.3***	1.2 ± 0.1*	9.1 ± 0.4***
	21	29.4 ± 0.9***	0.9 ± 0.1	12.4 ± 0.7**	12.0 ± 0.5*	25.1 ± 0.8***	2.5 ± 0.2	9.2 ± 0.5**	1.4 ± 0.1***	0.9 ± 0.1***	5.4 ± 0.2***
Tropical fish (9)	0	29.6 ± 1.0	1.1 ± 0.2	12.5 ± 0.4	11.7 ± 0.3	25.7 ± 1.2	2.9 ± 0.2	9.8 ± 0.7	0.8 ± 0.1	1.1 ± 0.1	3.9 ± 0.3
	14	32.7 ± 1.4*	1.7 ± 0.3*	10.1 ± 0.7***	11.4 ± 0.7	15.5 ± 0.6***	3.0 ± 0.3	15.7 ± 0.1***	1.2 ± 0.1*	1.2 ± 0.1	6.6 ± 0.5***
	21	30.7 ± 1.3	1.0 ± 0.2**	11.8 ± 0.6***	11.2 ± 0.4	25.8 ± 0.9***	2.5 ± 0.2***	10.4 ± 0.6***	0.8 ± 0.1**	0.8 ± 0.1***	4.3 ± 0.4***
Kangaroo (8)	0	28.8 ± 1.0	1.0 ± 0.1	12.1 ± 0.6	11.5 ± 0.6	26.4 ± 1.7	3.0 ± 0.2	10.8 ± 0.8	1.1 ± 0.1	1.1 ± 0.1	3.5 ± 0.4
	14	33.9 ± 1.4***	1.7 ± 0.3*	10.6 ± 0.6***	11.0 ± 0.6	16.3 ± 1.1***	3.6 ± 0.3*	15.1 ± 0.8***	1.4 ± 0.1*	1.5 ± 0.1***	4.0 ± 0.5
	21	29.7 ± 1.0*	1.1 ± 0.2*	12.1 ± 0.8	12.0 ± 0.7	23.5 ± 1.2***	3.3 ± 0.2*	12.4 ± 0.6***	1.4 ± 0.2	1.4 ± 0.1	3.8 ± 0.3
Vegetarian (10)	0	29.4 ± 0.7	1.4 ± 0.1	12.4 ± 0.4	12.8 ± 0.8	25.7 ± 0.7	2.9 ± 0.2	9.2 ± 0.6	0.9 ± 0.1	0.8 ± 0.1	3.4 ± 0.3
	14	34.8 ± 1.7**	2.0 ± 0.2*	11.2 ± 0.5*	12.8 ± 0.7	20.1 ± 1.5**	3.4 ± 0.4	9.7 ± 0.6	0.6 ± 0.1**	0.8 ± 0.1	3.8 ± 0.3
	21	30.5 ± 0.6*	1.6 ± 0.2*	12.4 ± 0.5*	12.9 ± 0.7	24.1 ± 1.0**	2.8 ± 0.2	9.0 ± 0.7	1.4 ± 0.3*	0.9 ± 0.1	3.2 ± 0.2
Cholesteryl esters											
Southern fish (10)	0	11.5 ± 0.6	3.3 ± 0.3	0.9 ± 0.1	18.1 ± 0.9	56.6 ± 1.4	0.7 ± 0.1	6.3 ± 0.6	0.9 ± 0.1		0.9 ± 0.1
	14	15.4 ± 0.7***	5.1 ± 0.6**	1.2 ± 0.4	21.4 ± 1.0*	40.2 ± 2.1***	0.9 ± 0.2	9.9 ± 0.7***	3.1 ± 0.5***		2.0 ± 0.1***
	21	12.1 ± 0.5***	3.2 ± 0.4**	0.9 ± 0.1	18.7 ± 1.0*	49.2 ± 6.3	1.4 ± 0.7	6.3 ± 0.5***	1.1 ± 0.1**		1.3 ± 0.1**
Tropical fish (9)	0	12.6 ± 0.8	3.8 ± 0.4	1.1 ± 0.1	17.9 ± 0.8	55.7 ± 1.8	0.7 ± 0.1	6.2 ± 0.8	0.8 ± 0.1		0.7 ± 0.1
	14	15.1 ± 0.8**	6.8 ± 0.9**	0.9 ± 0.1	21.1 ± 0.9**	40.4 ± 1.5***	1.0 ± 0.1*	11.3 ± 0.9***	1.0 ± 0.1		1.6 ± 0.3**
	21	11.9 ± 0.6***	3.8 ± 0.5***	1.0 ± 0.1	17.4 ± 1.0*	55.4 ± 1.9***	0.6 ± 0.1*	7.3 ± 0.7***	0.7 ± 0.1**		1.1 ± 0.2**
Kangaroo (8)	0	12.7 ± 0.8	3.8 ± 0.2	1.0 ± 0.2	19.2 ± 0.9	53.7 ± 1.8	0.7 ± 0.1	6.7 ± 0.7	0.9 ± 0.1		0.7 ± 0.1
	14	16.8 ± 1.5**	6.5 ± 0.7***	1.1 ± 0.1	20.9 ± 0.8	39.3 ± 2.1***	1.2 ± 0.1***	10.8 ± 0.1***	1.3 ± 0.1***		1.3 ± 0.3*
	21	13.2 ± 0.8**	4.5 ± 0.4***	1.0 ± 0.1	19.2 ± 1.0	50.3 ± 2.1***	0.9 ± 0.1*	8.1 ± 0.6**	1.1 ± 0.1		0.8 ± 0.1
Vegetarian (10)	0	12.3 ± 0.5	5.4 ± 0.8	1.2 ± 0.1	16.8 ± 1.1	55.6 ± 1.7	0.6 ± 0.1	5.4 ± 0.6	0.6 ± 0.1		0.7 ± 0.1
	14	13.9 ± 0.4*	7.6 ± 1.2**	1.3 ± 0.3	20.8 ± 1.5**	46.4 ± 2.5***	0.8 ± 0.1	6.7 ± 0.7**	0.5 ± 0.1		1.1 ± 0.3
	21	12.0 ± 0.5*	5.6 ± 1.0*	1.5 ± 0.1	18.8 ± 1.0	53.1 ± 1.8**	0.7 ± 0.1	5.5 ± 0.4*	0.8 ± 0.1*		1.0 ± 0.3

Statistical comparisons (relative to the value above): *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

[a]Baseline, day 0; diet, day 14; postdiet, day 21.

others showing reductions in both LDL and HDL cholesterol on diets that either are low in fat (10) or have a high P/S ratio (11). It appears that monounsaturated fatty acids (oleic acid as in olive oil) are unique in specifically lowering LDL cholesterol while leaving HDL cholesterol relatively unaffected (12). In the present study, consistent reductions in plasma cholesterol occurred rapidly in normo-cholesterolemic subjects consuming low fat diets with quite different cholesterol intakes (11–316 mg/day) and appear to be explained entirely by the very low fat contents.

High carbohydrate intakes are associated with increased triglyceride and reduced HDL-cholesterol concentrations (13). In the present study, all diets were associated with consistent falls in HDL-cholesterol, but there were no significant effects on triglycerides. In view of the well-documented triglyceride-lowering effect of fish and n-3 PUFA (14), it may be significant that the greatest trends toward increased triglycerides (not statistically significant) were observed on the two diets that did not contain fish (kangaroo and vegetarian). The relatively high dietary fiber intake (from fruit and vegetables) may have also helped prevent the rise in triglycerides (15).

Another consistent finding on all diets was the substantial reduction in the percentage of linoleic acid in both the cholesteryl ester and phospholipid fractions and the increase in the palmitic acid percentage in the phospholipid and cholesteryl ester fractions and palmitoleic acid percentage in the cholesteryl ester fraction. The reductions in linoleate were evident in all subjects within seven days of starting the low fat diets and were even more marked by 14 days. Following the resumption of the normal high fat diets the percentage of linoleate returned to the prediet values. The linoleic acid content of all the low fat diets was considerably reduced compared with the normal high fat diet values, and this presumably acounts for the reduction in the percentage of linoleic acid in the plasma lipids. Some workers have estimated that the dietary requirements of linoleic acid in man are greater than the values found on the present low fat diets (16); however, there was no evidence of any significant amounts of 20:3n-9 in the plasma in these studies.

There were other changes in plasma lipid fatty acid profiles in this study that were quite diet-dependent. The three diets containing 20- and 22-carbon PUFA showed a marked alteration of fatty acid profiles, with an obvious positive relationship between the presence of particular dietary 20- and 22-carbon PUFA and the increase in the percentage of those PUFA in the phospholipid and cholesteryl ester fractions. There was no change in the percentage of 20- and 22-carbon PUFA on the vegetarian diet, suggesting that the increase of these PUFA in the plasma lipids on the other low fat diets was due to their presence in the diet.

Despite very low levels of the dietary 20- and 22-carbon PUFA (0.3–1.1 g per day), there was a significant increase in the proportion of these fatty acids in the plasma lipids. The data also show that there is a marked difference in the incorporation of dietary 18-carbon PUFA and dietary 20-and 22-carbon PUFA in man. This is particularly evident on the kangaroo diet, where the level of 18:2n-6 was twice that of 20:4n-6 in the meat, yet the percentage of 20:4n-6 rose and that of 18:2n-6 fell in the plasma lipids. This difference has been noted previously in our field studies (2). Differences between the incorporation of dietary 20:4n-6 and 22:6n-3 relative to their respective 18-carbon precursors have been noted previously in experimental animals (17–20) and also more recently in man for 20:5n-3 and 22:6n-3 compared with 18:3n-3 (21).

Since the predominant PUFA in the Western diet are 18-carbon PUFA (22), it has been assumed that most of the tissue 20- and 22-carbon PUFA originate from the conversion of the 18-carbon EFA in the liver. However, there has been some discussion as to the relative importance of dietary long chain PUFA, since it has been argued that the conversion process of 18- to 22-carbon PUFA in man is an inefficient process (23–25). The present results using very low fat diets show that small amounts of dietary 20- and 22-carbon PUFA (0.3–1.1 g/day) can have a significant effect on the plasma fatty acid composition in man.

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Effect of Choline, Ethanolamine and Serine Supplementation on the Membrane Properties of *Microsporum gypseum*

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Phospholipid bases, choline and ethanolamine, when supplemented in the growth medium of *Microsporum gypseum* resulted in an increase in the corresponding phospholipid and total phospholipid content. However, when serine was supplemented, marginal changes were observed. The fatty acid profile of phospholipids remained almost unchanged. The uptake of lysine, leucine and aspartic acid in the spheroplasts of choline- and ethanolamine-grown cells was higher as compared to the control. 1-Anilinonaphathalene-8-sulfonate (ANS) binding to the spheroplast membrane, as calculated from Scatchard plots, demonstrated an increase in the number of binding sites in choline- and ethanolamine-grown cells, while a decrease was observed in the serine-supplemented cells. The results are discussed in terms of the effect of phospholipid polar head group composition on the membrane structure and function of this fungus.
Lipids 22, 530-534 (1987).

Several factors, such as growth, temperature and composition of the medium, have previously been utilized to modulate the phospholipid composition of the membranes of various microorganisms (1). Less attention has been focused on determining the exact role of lipids and phospholipids in the structure and function of dermatophyte membranes. Dermatophytes, a group of filamentous, pathogenic fungi, have been studied extensively for their lipid composition and lipid-metabolizing enzymes and phospholipid metabolism (2). Several antifungal drugs are known to bind the membrane components, namely, fatty acids, sterols and phospholipids (1,3,4); hence, it becomes essential to study the role of phospholipids in the structure and function of dermatophyte membranes. While information is available on the role of lipids in amino acid accumulation in dermatophytes, no attention has been given to the structural aspects of the membranes (5,6) as influenced by lipid content. In this study, different nitrogenous phospholipid bases (choline, ethanolamine and serine) have been used to modulate the phospholipid composition of a dermatophyte, *Microsporum gypseum*. These compounds have been known to enrich the cell membrane with corresponding phospholipids (phosphatidylcholine and phosphatidylethanolamine) in *Saccharomyces cerevisiae* and *Candida albicans* (7,8). Further attempts have been made to correlate the alterations produced in phospholipids with the structural and functional changes in the membranes.

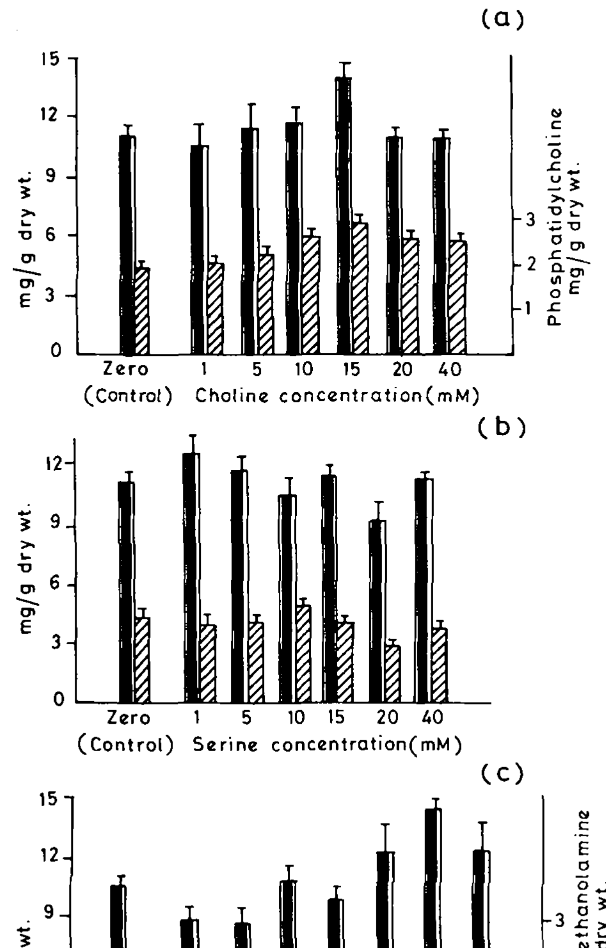

FIG. 1. Effect of supplementation of nitrogenous bases on the total (TPL) and individual corresponding phospholipid levels. (a) Effect of choline supplementation on TPL (solid column) and phosphatidylcholine (hatched column) levels; (b) effect of serine supplementation on TPL (solid column) and phosphatidylserine + phosphatidylinositol (PS+PI; hatched column) levels; and (c) effect of ethanolamine supplementation on TPL (solid column) and phosphatidylethanolamine (PE; hatched column) levels.

MATERIALS AND METHODS

Materials. Labeled amino acids, [U-^{14}C]L-lysine (sp act 126 mCi/mmol), [U-^{14}C]L-leucine (sp act 62 mCi/mmol) and [U-^{14}C]L-aspartic acid (sp act 152 mCi/mmol), were obtained from BARC (Bombay, India). Lysine, leucine and aspartic acids were purchased from Sigma Chemical Co. (St. Louis, MO). Novozyme 234 was from M/S Novo Industries (Bagsvaerd, Denmark). Ficoll-paque was purchased from Pharmacia Fine Chemicals Co. (Uppsala, Sweden). 1-Anilinonaphthalene-8-sulfonate (ANS), Mg salt, was obtained from Fluka (Buchs, Switzerland). Membrane

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Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

filters (0.45 μM) and a 12-place sampling manifold used for the amino acid accumulation experiment were obtained from Whatman Inc. (London, U.K.) and Millipore Inc. (Bedford, MA), respectively. All other chemicals used were of the highest purity available.

Growth of culture. M. gypseum, obtained from the Mycological Reference Laboratory, School of Hygiene and Tropical Medicine, (London, U.K.) was maintained on Sabouraud's dextrose agar slants. It was grown for experimental purposes in Sabouraud's broth (4% glucose, 1% peptone, pH 5.4–5.6) at 27 C as surface cultures. Choline, ethanolamine and serine were supplemented in the medium at different concentrations. The cells were harvested 16 days after inoculation in mid-log phase and processed for lipid extraction and membrane studies by the following methodology.

Quantitation of phospholipids. The lipids were extracted from the cells by Folch's extraction procedure (9). Total phospholipids were quantitated by the method of Bartlett, as modified by Marinetti (10). The individual phospholipids were separated by single dimension thin layer chromatography using the solvent system chloroform/methanol/7N NH_3 (65:25:4,v/v/v). Total phospholipids separated from the neutral lipids were used for fatty acid analysis. Fatty acids were converted into their methyl esters by transesterification with methanol in the presence of thionyl chloride, as described earlier (11). The methyl esters were separated on a Pye Unicam Model 104 Gas Chromatograph, using a flame ionization detector and column containing 10% diethylene glycol succinate on CAW (100–200 mesh) with nitrogen as a carrier gas at a flow rate of 40 ml/min. The fatty acids were quantitated by triangulation.

Preparation of spheroplasts. Spheroplasts were prepared by the procedure of Larroya et al. (12) with some modification. Log phase cells were incubated under sterile conditions with Novozyme 234 (30 mg/g wet wt of mycelium) in a conical flask in 10 mM citrate phosphate buffer (pH 6.5) containing 145 mM NaCl for 12–15 hr in a shaker at 30 C. Formation of spheroplasts was monitored microscopically. The incubation mixture was removed by centrifugation at 1000 g for 10 min to recover the cells. The pellet was washed twice with citrate phosphate buffer as mentioned above. The spheroplasts were

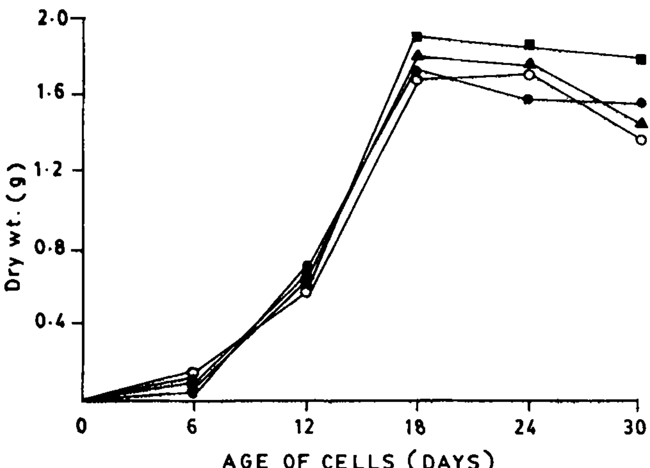

FIG. 2. Effect of supplementation of nitrogenous bases (at optimum concentration) on the growth pattern of *M. gypseum*.

purified by centrifugation on a Ficoll-paque density gradient at 400 g for 15 min. The spheroplast fraction, free of cell debris, was used for further studies (amino acid uptake and structural studies with fluorescent probe ANS).

Amino acid uptake studies. Amino acid uptake by spheroplast was examined according to a method standardized in our laboratory. In brief, spheroplasts containing 100–150 μg protein were incubated at 27 C for 7 min (optimum time) in 10 mM citrate phosphate buffer (pH 6.5) containing 145 mM NaCl; 800 nmol of labeled amino acid (sp act 0.2 μCi/μmol) was added to bring the total assay volume to 200 μl. The Kt values for lysine, leucine and aspartic acid were found to be 2.0, 2.3 and 2.8 mM, respectively. The reaction was started by the addition of respective amino acid, stopped by the addition of chilled buffer and rapidly filtered through 0.45 μM membrane filters. The filter paper was washed twice, dried and counted for radioactivity in a toluene-based fluid in a Packard Tricarb Liquid Scintillation Counter. Protein was estimated by the method of Lowry et al. (13).

Structural studies with ANS. ANS was used to study the structural changes in the spheroplast membrane pre-

TABLE 1

Effect of Nitrogenous Bases (at Optimum Concentration) on the Phospholipids of *M. gypseum*

	Nitrogenous base supplemented (mg/g dry wt of cells)			
	None (control)	Choline (15 mM)	Serine (10 mM)	Ethanolamine (40 mM)
TL	103.80 ± 7.50	135.90 ± 4.60*	126.00 ± 15.10†	89.00 ± 6.66†
TPL	11.15 ± 0.60	13.80 ± 0.60*	10.48 ± 0.40†	14.50 ± 0.45**
LPC	2.05 ± 0.30	1.60 ± 0.20†	1.85 ± 0.24†	2.64 ± 0.22†
PS+PI	4.25 ± 0.40	5.90 ± 0.50†	4.65 ± 0.31†	4.87 ± 0.21†
PC	1.96 ± 0.17	2.92 ± 0.20*	2.01 ± 0.18†	3.10 ± 0.35**
PE	1.36 ± 0.12	1.89 ± 0.40†	0.82 ± 0.08*	2.25 ± 0.11**
Unidentified PL	1.61 ± 0.19	1.48 ± 0.30†	1.78 ± 0.33†	1.63 ± 0.13†
LPC + PC + PE/(PS + PI)	1.26	1.10	1.01	1.64

Mean ± S.E. of four independent batches in duplicate. *, $p < 0.05$; **, $p < .01$; †, nonsignificant. TL, total lipid; TPL, total phospholipid; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

TABLE 2

Effect of Nitrogenous Bases (at Optimum Concentration) on the Fatty Acid Composition of *M. gypseum* Phospholipids (Relative Percentage)

Nitrogenous base supplemented	Fatty acids						
	14:0	14:1	16:0	18:0	18:1	18:2	U/S
None (control)	8.66 ±2.06	1.06 ±0.21	24.90 ±3.21	11.70 ±2.53	18.70 ±2.05	35.54 ±2.98	1.20
Choline (15 mM)	11.69† ±1.74	—	28.29† ±2.98	11.29† ±2.05	11.10* ±1.91	37.63† ±1.47	0.95
Serine (10 mM)	7.65† ±1.48	1.78† ±0.22	30.50† ±1.71	8.72† ±0.98	16.09† ±1.53	35.20† ±2.85	1.13
Ethanolamine (40 mM)	8.48† ±1.87	tr	27.73† ±1.99	12.54† ±2.38	11.93** ±1.05	40.50† ±2.62	1.10

Mean ± SE of four batches in duplicate. *, $p<0.05$; **, $p<0.01$; †, nonsignificant; U, unsaturated; S, saturated; tr, trace.

TABLE 3

Effect of Nitrogenous Base Supplementation (at Optimum Concentration) on the Uptake of Amino Acids in Spheroplasts Prepared from *M. gypseum* Cells

Base	Amino acid uptake		
	L-Lysine	L-Leucine	L-Aspartic acid
None (control)	6.12 ± 1.83	6.50 ± 1.72	18.76 ± 1.79
Choline (15 mM)	13.06 ± 2.66***	10.99 ± 3.75**	26.70 ± 2.73***
Serine (10 mM)	7.48 ± 1.68†	8.08 ± 2.04†	19.92 ± 2.54†
Ethanolamine (40mM)	13.16 ± 3.40**	10.94 ± 4.20**	24.93 ± 1.39***

Uptake in nmol/mg protein/7 min. Mean ± S.D. of 8–11 independent observations. **, $p<0.01$; ***, $p<0.001$; †, nonsignificant.

pared from variously grown *M. gypseum* cells. The basic assay mixture consisted of a total volume of 1 ml in 10 mM citrate phosphate buffer (pH 6.5) containing 145 mM NaCl, 50 μM ANS and spheroplast protein ranging from 20–250 μg. The readings were taken as an emission spectrum in a Shimadzu RF-540 spectrofluorimeter with a recorder. Quartz cuvettes of 1.5 ml capacity and 0.5 cm light path were used for the study. Zero correction and spectrum correction were applied and the number of binding sites were calculated from the Scatchard plots (14) drawn as described elsewhere (15).

RESULTS AND DISCUSSION

Phospholipids of the cell are almost exclusively present in the cellular membranes in most microorganisms. Any change in the growth medium or conditions is likely to result in alterations in the normal structure and function of the membrane. The phospholipid composition can be altered in vivo, either (i) by inclusion of fatty acids and other additives in the medium, which results in an altered fatty acyl chain composition or (ii) by including the precursors of phospholipid polar head groups in the growth medium (16). In this investigation, we have followed the latter approach to examine the influence of polar head groups of phospholipids on the membrane functions in *M. gypseum* cells, as the first approach has already been adapted for this fungus (17).

Phosphatidylserine (PS) plus phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were found to be the major phospholipids in *M. gypseum*; this differed from our earlier reports (5,6) because of age of the culture, separation techniques and composition of the medium. Optimum concentrations for the supplementation of the nitrogenous phospholipid bases, namely, choline, serine and ethanolamine, were determined by measuring the levels of total and corresponding phospholipids. As PI was a minor component with mobility close to PS, they were pooled. Figure 1 shows that 15, 10 and 40 mM concentrations of choline, serine and ethanolamine, respectively, were found to be optimum concentrations, as total phospholipid and corresponding phospholipid content has maximum alterations at these concentrations vs the control where no additive was supplemented in the medium. However, the increase in PS + PI on serine supplementation was marginal. The *M. gypseum* cultures grown in the medium supplemented

with nitrogenous bases did not demonstrate any difference in the growth pattern (Fig. 2), as observed earlier with *S. cerevisiae* (7). Hence, cells were harvested in the mid-log phase after 16 days of growth. Supplementation of the choline analogues like N,N-dimethylethanolamine, N-monomethylethanolamine and ethanolamine also did not significantly affect the growth of C_6 glial cells in culture (16).

Total phospholipid levels increased significantly with choline and ethanolamine, but no significant change was observed with serine (Table 1). Supplementation of choline and ethanolamine has also been demonstrated to enhance the total phospholipid content in *S. cerevisiae* (7). The levels of PC and PE increased significantly on ethanolamine supplementation, although only PC content increased when choline was supplemented. The levels of other phospholipids were not altered significantly under these conditions. The increase in the PS + PI fraction on serine supplementation was not significant, but PE levels decreased significantly. In *C. albicans* and *S. cerevisiae,* choline and ethanolamine supplementation altered the PC and PE content, respectively (1,8), identical to our observations with *M. gypseum.*

The fatty acid profile, as well as the ratio of unsaturated to saturated (U/S) fatty acids, did not change significantly with supplementation of these nitrogenous bases (Table 2). However, a significant decrease (40%) was observed in oleic acid content with supplementation of choline and ethanolamine. The insignificant changes observed in phospholipid fatty acids provide a system to study the functional role of phospholipid polar head groups in membranes of *M. gypseum.* Similar observations have been reported with C_6 glial cells (16) where supplementation of polar head groups resulted in alteration in the phospholipids, but no change was seen in the fatty composition. In the absence of any major change in fatty acid composition and enrichment of methylated phospholipids like PC, it is expected that choline- and ethanolamine-supplemented cells will have more permeable membranes (18,19).

The functional properties of the membranes in response to changes in phospholipid composition were studied by measuring the uptake of a basic, neutral and acidic amino acid under optimal conditions. The uptake of all three amino acids (lysine, leucine and aspartic acid) increased in the spheroplasts prepared from choline- and ethanolamine-grown cells about 1.5- to 2-fold, while in serine-supplemented cells, it did not change significantly (Table 3). There have been various reports on the functional aspects of polar head group–induced alterations in the phospholipids and the rigidifying or fluidizing effects on the membrane have been demonstrated. In *C. albicans* and *S. cerevisiae,* choline and ethanolamine supplementation modulates the phospholipid composition in such a way that the amino acid uptake is inhibited in supplemented cells, irrespective of the variations in the PC/PE ratio (8). The amino acid uptake alters with a change in the carbon source, addition of ethanol or alteration of growth temperature (6). Although the PC/PE ratio plays a crucial role in the functioning of the membrane, there are various other components such as proteins, glycoproteins and sterols that have an overall combined effect on the membrane structure and functions.

Changes in the phospholipid composition and function have not been correlated with the structural organization of the membranes. There have been reports on the effect of supplementation of the media with choline and ethanolamine, but no information is available with serine. The Scatchard plot analysis shows increased binding sites in spheroplasts prepared from choline- and ethanolamine-supplemented cells (Fig. 3). Trivedi et al. (8) have demonstrated a decrease in the number of binding sites with choline and ethanolamine supplementation in two different strains of *S. cerevisiae,* but our results showed an increase in *M. gypseum,* although the number in serine-grown cells was lower vs unsupplemented cells. As is apparent from the phospholipid composition of vari-

TABLE 4

Effect of Supplementation of Phospholipid Bases on the Number of Binding Sites for ANS[a] on *M. gypseum* Spheroplasts

Type of cells (base supplemented)	No. of binding sites (nmol/mg protein)
None (control)	180
Choline (15 mM)	240
Serine (10 mM)	152
Ethanolamine (40 mM)	198

[a]ANS, 1-anilinonaphthalene-8-sulfonate.

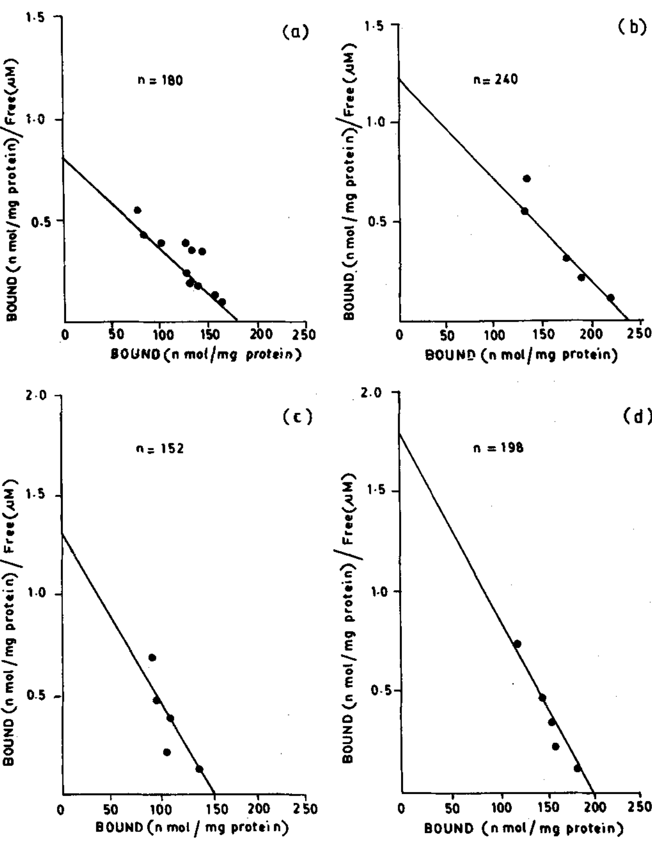

FIG. 3. Scatchard plots of 1-anilinonaphthalene-8-sulfonate binding with *M. gypseum* spheroplasts (based on experiments carried out in duplicate) of (a) control cells; (b) choline-grown cells; (c) serine-grown cells; and (d) ethanolamine-grown cells.

ously grown cells (Table 1), the levels of PC increased in choline-supplemented cells and both PC and PE increased in ethanolamine-grown cells, but PE decreased in serine-supplemented cells. At physiological pH, PE is slightly negatively charged compared to PC, which is neutral and exists in zwitterionic form (20). Hence, the binding of ANS, which itself is anionic at neutral pH, would be increased in membranes having more PC than PE. Thus, high PC content in choline- and ethanolamine-grown cells may be responsible for the increase in the number of binding sites in these cells vs unsupplemented cells. Similarly, the decrease in the number of binding sites in serine-grown cells may be due to another anionic component, PS + PI, which has a role in the overall membrane surface charge. Lower phospholipid content in serine-supplemented cells of *M. gypseum* may also be the reason for the observed decrease in the number of binding sites in these cells. The changes observed in the ANS binding pattern may also be due to either the changed hydrophobic environment around the embedded dye or the altered hydrophobic environment around the integral membrane proteins (21,22). It has been shown that ANS does not enter the cell interior and is mainly bound to the membrane surface (23); hence, the observed changes demonstrate a role of phospholipids and proteins in cell membrane.

In brief, it can be concluded from this study that supplementation of phospholipid bases results in the enrichment of PC or PE in the membranes of *M. gypseum* without inducing any significant alteration in the fatty acid composition of phospholipids. Functional and structural changes could be correlated with the phospholipid polar head group composition.

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Slow Recovery of the Fatty Acid Composition of Sciatic Nerve in Rats Fed a Diet Initially Low in n-3 Fatty Acids

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The sciatic nerve of rats fed sunflower oil (6 mg 18:3-n-3/100 g of diet) presented dramatic alterations in the long chain polyunsaturated fatty acids in comparison with those fed soy oil (130 mg 18:3n-3/100 g of diet). In both 15-day-old and 60-day-old animals fed sunflower oil, 22:6n-3 (cervonic acid) was four-fold less, 22:5n-6 was 10-fold greater; adrenic acid (22:4n-6) was slightly greater and arachidonic acid (20:4n-6) was close to that in rats fed soy oil. The percentage distribution of total polyunsaturated fatty acids as well as the individual saturated and monounsaturated fatty acids were the same in both groups.

When the sunflower oil–fed animals were switched to a soy oil–containing diet for either 15 or 60 days, the percentage distribution of 22:6n-3 increased slowly to reach the control value 2.5 months later. Conversely 22:5n-6 decreased slowly. The decay of 22:5n-6 was more rapid than the increase of 22:6n-3.
***Lipids 22,* 535–538 (1987).**

The fluidity of the lipid environment appears to modulate the activity of membrane-bound enzymes; this fluidity is controlled by essential polyunsaturated fatty acids (PUFA). Increased PUFA, particularly arachidonic acid, were found in endoneurial phosphatidylethanolamine of both developing and regenerating rat sciatic nerve, suggesting a close association between PUFA and peripheral nerve myelination (1). Alterations of PUFA during damage paralleled changes in phospholipid fatty acid composition (2).

The effect of essential fatty acid (EFA) deficiency on rat peripheral nerve myelin has been previously analyzed (3). After 8 mo on the deficient diet, 20:3n-9 was found in the major myelin phospholipids. The level of 18:1n-9 was increased and the levels of 18:2n-6 (linoleic acid), 20:4n-6 (arachidonic acid) and 22:4n-6 (adrenic acid) were decreased. The ratio of 20:4n-6 to 20:3n-9 was clearly depressed by an EFA deficiency (3).

No significant differences in morphology, histology, rate of axonal transport or conduction velocity of peripheral nerve were demonstrated between EFA deficient and control rats (4). Therefore, it would appear that dietary EFA deficiency in postweaned rats can induce fatty acid alterations in peripheral nerve myelin without resulting in detectable changes in function or structure. An excellent review of the lipid composition of normal and degenerating nerve has been recently published (5).

The essentiality of PUFA for the central nervous system has been extensively studied (6–14). The specific role of n-3 fatty acids has been recently reexamined. Interestingly, a pathogenesis of deficiency in linolenic acid has

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TABLE 1

Diet Composition (g/kg)

	Soy oil diet	Sunflower oil diet
Casein delipidated[a]	220	220
DL-methionine	1.6	1.6
Cellulose	20	20
Starch	459.7	463.4
Saccharose	230	230
Oil	18.7	15.0
Vitamin mixture[a]	10	10
Mineral mixture[b]	40	40

[a]United States Biochemical Corp. (Cleveland, OH). The vitamin mixture used is the vitamin diet for fortification mixture.
[b]Composition of the mineral mixture/100 g: $CaHPO_4$, 2 H_2O, 38.0: K_2HPO_4, 24.0; $CaCO_3$, 18.1; NaCl, 7.0; MgO, 2.0; MgSO4, 7 H_2O, 9; $FeSO_4$, 7 H_2O, 0.7; $ZnSO_4$, 7 H_2O, 0.5; $MnSO_4$, H_2O, 0.5; $CuSO_4$, 5 H_2O, 0.1; NaF, 0.1; $Al_2(SO_4)_3$ K_2SO_4, 24 H_2O, 0.02; KI, 0.008; $CoCO_3$, 0.008; Na_2SeO_3, 5 H_2O, 0.001.

TABLE 2

Fatty Acid Composition (mg %) of Dietary Lipids

Fatty acids	Sunflower oil diet	Soy oil diet
14:0	0.3	0.3
16:0	6.4	10.1
17:0	tr	0.2
18:0	3.9	5.6
20:0	0.3	0.4
22:0	0.7	0.5
Total saturated	11.6	17.2
16:1	0.2	tr
18:1	21.4	21.4
20:1	0.2	0.3
Total monounsaturated	21.6	21.7
18:2 n-6	66.4	53.5
18:3 n-3	0.4	7.4
Fatty acids (mg/100 g) of diet		
18:2 n-6	936.0	940.0
18:3 n-3	6.0	130.0
n-3/n-6	0.006	0.14

tr, Traces. Dietary lipid fatty acid composition was analyzed by gas chromatography of methyl esters under the following conditions: Packard model 427 gas chromatograph; glass capillary column; stationary phase FFAP; gas pressure vector H_2, 0.6 bar; temperature, 190 C; detection by flame ionization.

been described in the monkey (15) and in man (16). A syndrome of modern society has been put forward as a deficiency in acids of the n-3 series (17).

Our previous studies have shown that a diet lacking in α-linolenic acid (sunflower or peanut oil) can modify the PUFA composition in all the cellular and subcellular fractions of brain lipids examined. The percentage distribution of total PUFA remains unchanged, the sharp drop in 22:6n-3 being counterbalanced by an increase in 22:5n-6 (18,19). With a change from a diet containing sunflower oil to one containing soy oil, the uptake of n-3 fatty acids is remarkably slow, e.g., it takes several months before the cerebral organelles recover a normal quantity of cervonic acid (22:6n-3) (20).

However, little attention has been paid to n-3 PUFA in the peripheral nerve. This work was undertaken to determine (i) the specific alterations of peripheral nerve fatty acids in rats fed a diet rich in n-6 fatty acids but deficient in n-3 fatty acids and (ii) to assess the speed of the recovery of the n-3 fatty acid composition induced when a diet containing n-3 fatty acids is reintroduced.

Our preliminary results have shown that when rats are fed a diet deficient in n-3 fatty acids (peanut or sunflower oil), both peripheral nerve and muscle contain reduced amounts of n-3 fatty acids; more specifically, 22:6n-3 is replaced by 22:5n-6 (21).

MATERIALS AND METHODS

Animals. During three generations, female Wistar rats

TABLE 3

Fatty Acid Percentage Distribution (mg %) of Sciatic Nerve from Young and Adult Rats Fed a Diet Containing Either Soy Oil or Sunflower Oil

	Sunflower oil		Soy oil	
Fatty acid	15 days	60 days	15 days	60 days
Saturated	46.0	40.5	45.3	40.8
14:0	4.0	0.9	4.0	1.5
15:0	0.1	0.1	0.1	0.2
16:0	28.2	23.1	27.1	27.8
17:0	0.2	0.2	0.2	0.2
18:0	8.4	8.2	8.4	6.3
20:0	0.9	1.5	0.9	0.8
22:0	1.8	3.2	1.8	1.9
23:0	0.3	0.4	0.3	0.2
24:0	2.0	2.6	2.2	1.7
25:0	0.1	0.3	0.3	0.2
Monounsaturated	37.6	47.6	39.0	49.0
16:1n-9	0.8	0.8	0.8	0.4
16:1n-7	2.0	3.2	2.1	8.4
18:1n-9	26.1	32.2	28.0	33.1
18:1n-7	2.7	3.1	2.7	3.1
20:1n-9	0.6	0.8	0.6	0.4
20:1n-7	0.7	1.0	0.8	0.8
22:1n-9	0.3	0.5	0.3	0.2
22:1n-7	0.3	0.4	0.2	0.2
24:1n-9	3.7	5.0	3.6	2.2
24:1n-7	0.4	0.6	0.4	0.2
n-9	31.5	39.3	33.8	36.3
n-7	6.1	8.3	6.2	12.7
Polyunsaturated	14.7	11.4	15.1	10.0
18:2n-6	2.9	2.8	3.0	5.0
20:3n-6	0.9	0.4	0.9	0.4
20:4n-6	6.8	3.8	6.5	2.0
22:4n-6	2.2	2.3	1.5	0.7
22:5n-6	1.1	1.0	0.2	0.1
24:4n-6	0.1	0.2	0.2	0.1
24:5n-6	0.3	0.4	0.2	tr
n-6	14.3	10.9	12.5	8.3
18:3n-3	tr	0.2	0.2	0.4
22:5n-3	tr		0.5	0.2
22:6n-3	0.4	0.3	1.4	1.1
n-3	0.4	0.5	2.1	1.7
n-3/n-6	0.03	0.05	0.17	0.21

In each experiment, fatty acid determination was performed in triplicate on 8 pooled nerves. At least 3 experiments were performed.

were fed with a semisynthetic diet containing 1.5% sunflower oil (6 mg % α-linolenic acid). Two weeks before mating, one group (2/3 of the animals) was continued on that diet, while the other group was changed to a diet in which sunflower oil was replaced by 1.9% soy oil (130 mg % α-linolenic acid). Both oils and diets contained ca. 940 mg n-6 fatty acids. Since animals fed either diet ate similar amounts of food, they ate the same amount of n-6 fatty acids. Soy oil–fed rats received ca. 22 times more n-3 fatty acids than animals fed sunflower oil. The composition of the oils and the diets is shown in Tables 1 and 2. Under these experimental conditions, the α-linolenic acid deficiency (sunflower oil–fed rats) had no effect on fecundity (% of pregnant females), fertility (number of pups/litter), pup birth weight, food intake and weight of pregnant or lactating females, or pup growth during suckling. However, this deficiency did cause abnormally high rates of perinatal mortality from birth to postpartum day 3 (22). Three days after delivery the litters (fourth generation) were adjusted to 10 animals. After weaning, the young rats received the same diet as their mother. At 15 days of age, half the animals fed sunflower oil were fed soy oil from then on. In these animals, the n-3–deficient diet was therefore substituted by an n-3–normal diet. The same procedure was performed on other animals at 60 days. Thus, we determined the speed of recovery of fatty acid composition in young and in adult animals. Only male animals were used throughout. The sciatic nerves were very carefully dissected to avoid any contamination by adipose tissue.

Analytical methodology. The sciatic nerve was dissected outward, and lipids were extracted by sonication in chloroform/methanol (2:1, v/v) (23,24) and methylated (25). Fatty acid methyl esters were separated by gas liquid chromatography (GLC) on an open tubular capillary column coated with FFAP (0.30 mm in diameter, 45 m long), using a flame ionization detector. Identification of fatty acids was performed with commercial standards by the means of relative retention times. Areas were calculated with an ICAP integrator (LTT, Paris, France).

RESULTS AND DISCUSSION

Fatty acid analysis (Table 3). The sciatic nerve of sunflower oil–fed animals (6 mg % n-3 fatty acids) presented dramatic alterations in the very long chain polyunsaturated fatty acids in comparison to soy oil–fed (130 mg % n-3 fatty acids) animals. The n-3/n-6 ratio was six- and four-fold less in 15- and 60-day-old animals fed sunflower oil. In both 15-day-old and 60-day-old animals, 22:6n-3 was ca. five- and four-fold less, respectively; conversely, 22:5n-6 was 10-fold greater in sunflower oil–fed animals (22:4n-6 was greater; 20:4n-6 was close to normal). The percentage distribution of PUFA to total fatty acids was normal in sunflower oil–fed animals, as were individual saturated and monounsaturated fatty acids.

In agreement with Yao (30), we found that during development 20:4n-6 and 22:6n-3 decreased and 18:1n-9 increased. The values we obtained compared favorably with previously published results (26–32).

A control group was obtained by feeding animals with regular diet (standard chow). No significant difference was found between this group and animals fed with soy oil.

This study was performed on whole nerve, and contami-

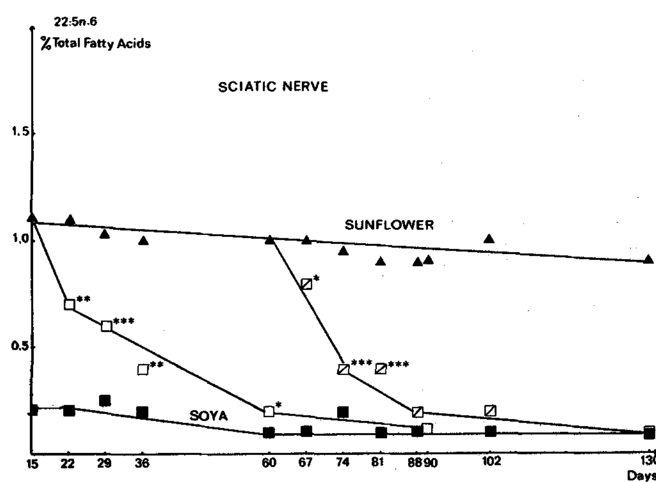

FIG. 1. Recovery of fatty acid composition (mg %) as measured by the amount of cervonic acid (22:6n-3) in animals fed soy oil, sunflower oil or soy oil replacing sunflower oil in either 15-day-old or 60-day-old rats. Dietary lipid fatty acid composition was analyzed by gas chromatography of methyl esters under the following conditions: Packard model 427 gas chromatograph; glass capillary column; stationary phase FFAP; gas pressure vector H_2, 0.6 bar; temperature, 190 C; detection by flame ionization. Solid squares, soy-fed animals; triangles, sunflower-fed animals; open squares, animals initially fed sunflower oil, then fed soy oil when 15 days old; hatched squares, animals initially fed sunflower oil, then fed soy oil when 60 days old. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$; 552 animals were used. Values at each time point represent mean for at least 4 samples. Each sample consisted of sciatic nerves from 4 rats.

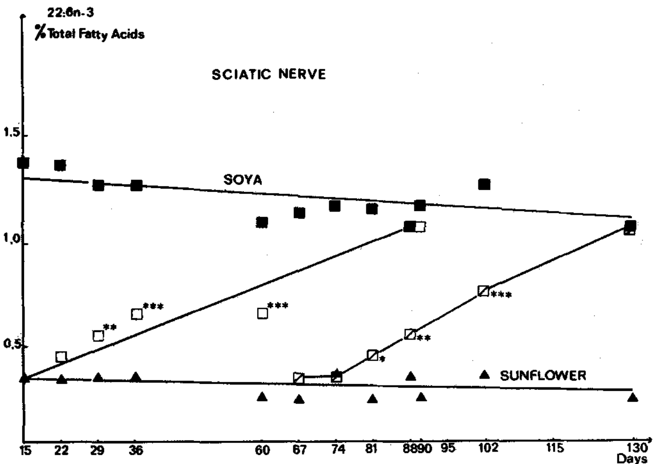

FIG. 2. Recovery of fatty acid composition (mg %) as determined by the amount of 22:5n-6 in animals fed soy oil, sunflower oil or soy oil replacing sunflower oil in either 15-day-old or 60-day-old rats. Dietary lipid fatty acid composition was analyzed by gas chromatography of methyl esters under the following conditions: Packard model 427 gas chromatograph; glass capillary column; stationary phase FFAP; gas pressure vector H_2, 0.6 bar; temperature, 190 C; detection by flame ionization. Solid squares, soy-fed animals; triangles, sunflower-fed animals; open squares, animals initially fed sunflower oil, then fed soy oil when 15 days old; hatched squares, animals initially fed sunflower oil, then fed soy oil when 60 days old. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$; 552 animals were used. Values at each time point represent mean for at least 4 samples. Each sample consisted of sciatic nerve from 4 rats.

nation by adipose tissue was carefully eliminated. However, endoneurium, perineurium and epineurium were not separated. Thus, specific alteration in one of these elements is not excluded as they present differences in lipid composition (5,32).

Recovery of altered fatty acid composition. Figure 1 shows changes for 22:6n-3 and Figure 2 for 22:5n-6 after changing the dietary oil from sunflower to soy oil. As no significant differences were found for the other fatty acids, the curves are not shown. However, 22:4n-6 was found to be increased in sunflower oil–fed animals, and the recovery after starting soy oil was slow in 60-day-old animals. In the 15-day-old animals, the decrease was hardly significant, and thus the recovery was difficult to estimate. The high level of 22:4n-6 in sciatic nerve in comparison with brain is to be noted. Sciatic nerves of adult animals fed sunflower oil contained a slightly increased percentage distribution of saturated and monounsaturated very long chain fatty acids. When the sunflower oil–fed animals received a diet containing soy oil at either 15 or 60 days, the percentage distribution of 22:6n-3 increased slowly to reach the control value after 2.5 months (Fig. 1). Conversely, 22:5n-6 decreased slowly throughout (Fig. 2). Interestingly, in adult animals, the recovery did not start from the day on which the diet was changed, but there was a delay of 20 days. In contrast, in young animals, the recovery started from a few days after changing the diet, but the recovery was statistically significant only after 14 days. It is interesting to note that the recovery as measured by the decay of 22:5n-6 was more rapid than with increase of 22:6n-3. This very low speed of recovery of PUFA composition of sciatic nerve after deprivation of n-3 fatty acids was unexpected. We have previously shown (20) that all brain subfractions (myelin, synaptosomes, mitochondria and microsomes) recover very slowly but all at the same speed. Thus, the peripheral nervous system responds to dietary fatty acid changes in a manner similar to the central nervous system.

The slow recovery could be due to the slow turnover of major membranes in the peripheral nervous system. Another explanation could be that the rate-limiting factor is the reduced in situ synthesis of n-3 fatty acid synthesis from α-linolenic acid due to low desaturase activity.

In addition, nervous tissue contains a very minute amount of α-linolenic acid, and studies of brain cells in culture have suggested that the only truly essential fatty acid for these cells is probably 22:6n-3 (33). Thus, another explanation for the low recovery of fatty acid composition could be either a limited transport through the blood-nerve barrier or a restricted synthesis of 22:6n-3 by the liver or reduced dietary origin.

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METHODS

An Improved Method for the Colorimetric Assay of Lipase Activity Using an Optically Clear Medium

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Lipase activity can be spectrophotometrically measured in an optically clear medium using long chain fatty thioesters of 1-mercapto-2,3-propanediol or 2-mercaptoethanol as substrates. With hexamethylphosphoric triamide solutions of these thiosubstrates, the Michaëlis-Menten constants of lipase from *Rhizopus arrhizus* were determined. The effects of calcium chloride and of bovine serum albumin on the enzyme activity were established.
Lipids 22, 539-541 (1987).

A surprisingly large number of different procedures has been reported for determining the activity of various lipase preparations (see recent review by Jensen [1]). This could imply that many of the present methods are not entirely satisfactory.

The most frequently used procedures are based on titrimetric determination of the acids liberated by the action of the enzyme, using either water-soluble or water-insoluble esters as substrates (2-5). Higher sensitivity than that of the titrimetric assay can be obtained using radioactively labeled substrates (6,7), but these procedures, of course, require the availability of labeled compounds.

A very interesting method for measuring lipases and phospholipases uses the bioluminescent assay (8). This method, however, involves a second enzymatic reaction (luciferase), the pH dependence of which is quite different from that of the lipase assay.

The colorimetric assay based on the development of the characteristic color of p-nitrophenol due to hydrolysis of its colorless esters provides a sensitive test of esterase activity (9). However, because of the low solubility of long chain fatty acid esters, the method is preferably applicable to esterase. The use of thioesters (Fig. 1) of fatty acids, associated with Ellman's reagent (10), has been investigated (11,12). Unfortunately, these thioesters are water-insoluble and require dispersing agents and special treatment before the colorimetric analysis (13,14).

We report here on an improved method using an optically clear medium for the colorimetric assay of lipase activity with long chain fatty acid thioesters.

MATERIALS AND METHODS

Reagents. Lipase of *Rhizopus arrhizus* was purchased from Gist-Brocades (Seclin, France) and used without further purification. Hexamethylphosphoric triamide (HMPA) and 5,5′-dithio-*bis*(2-nitrobenzoic) acids (DTNB) were obtained from Aldrich France (Strasbourg, France). All other chemicals were reagent grade, obtained from commercial sources.

Palmitoyl chloride. One hundred g (0.39 mol) of palmitic acid and 100 g (0.84 mol) of thionyl chloride were refluxed overnight. After removing the excess thionyl chloride under vacuum, the residue was distilled to give 93.8 g (0.35 mol) of colorless liquid (bp 18, 150 C).

Thioglycerol S-palmitate (1). 1-Mercapto-2,3-propanediol (10.8 g, 0.1 mol) and dry pyridine (5 g, 0.06 mol) were dissolved in 100 ml of anhydrous diethyl ether. The solution was cooled to 0 C, and 7 g (0.025 mol) of palmitoyl chloride in 30 ml of anhydrous diethyl ether was added dropwise. The mixture was left to stir at room temperature for 2 hr, then was washed four times with 50 ml of cold water and dried over sodium sulfate. Fifteen g of crude product containing a mixture of different acylated compounds were chromatographed on silica gel 60 (Merck, Darmstadt, Federal Republic of Germany; petroleum ether/diethyl ether, 90:10, v/v). The pure thioester obtained has physical properties identical to those described previously (11): IR ($CHCl_3$) 3400 (broad), 2940, 2870, 1700, 1460, 1080 cm^{-1}; mp, 78 C.

Mercaptoethanol S-palmitate (2). Dry pyridine (5 g, 0.06 mol) was added to a solution of 2-mercaptoethanol (8 g, 0.1 mol) in 100 ml of dry diethyl ether. Palmitoyl chloride (7 g, 0.025 mol) in 300 ml of dry diethyl ether was added dropwise at 0 C. The reaction mixture was left to stir at room temperature, then was washed four times with 50 ml of water and dried over sodium sulfate. Ten g of crude material was obtained after removing the organic solvent under vacuum. After being chromatographed on silica gel 60 (Merck; petroleum ether/diethyl ether, 90:10, v/v), 3.2 g (9.7 mmol) of pure product were isolated. IR ($CHCl_3$) 3490 (broad), 2950, 2860, 1690, 1460, 1110 cm^{-1}, mp, 55 C.

Enzyme activity assay. The measuring cuvette contained 100 μl of a HMPA thiosubstrate solution, 100 μl of a DTNB solution (initial concentration, 20 mg/ml in HMPA), 300 μl of HMPA and 2.4 ml of Tris buffer (initial concentration, 0.05 M; pH 8). The reaction mixture was kept at 37 C, and 100 μl of aqueous lipase solution was added. The absorbance was recorded immediately at 412 nm ($\varepsilon = 14.8\ 10^3\ l.mol^{-1}.cm^{-1}$) for 2 to 3 min. A linear increase of the absorbance was observed during this recording time.

$CH_2{-}SCO(CH_2)_{14}{-}CH_3$
|
$CH{-}OH$
|
$CH_2{-}OH$

1

$CH_2{-}SCO(CH_2)_{14}{-}CH_3$
|
CH_2OH

2

FIG. 1. Thioesters used as substrates.

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RESULTS

Due to their insolubility in water, compounds 1 and 2 cannot be used directly in the assay medium. In the past, this problem was overcome through use of a biphasic system and emulsifiers (14). In our approach, we searched for hydrophilic organic solvents. It is known that lipases can function in an aqueous medium containing up to 20% of organic solvent, and recently Zaks and Klibanov reported that lipases can maintain their activity also in anhydrous organic solvents (16). HMPA solutions of compounds 1 and 2 give an optically clear medium and develop a yellow color in the presence of lipase activity.

We studied the influence of different amounts of HMPA in the assay solution. As can be seen in Figure 2, maximum activity was obtained for 15% (v/v) HMPA. At this concentration, the medium remained optically clear.

We recorded the initial velocity obtained for thioesters 1 and 2 when the substrate concentration was greater than that of the enzyme. In Figure 3, the data are recorded in a log/log scale because of the large amplitude of the enzyme concentration. Both thioesters show a straight line obeying the equation

$$\log v = \log [E] + B$$

$$\text{where } B = \log K : v = K [E]$$

Based on kinetic measurements with these compounds, Michaëlis-Menten constants were determined (Table 1 and Fig. 4).

When one compares the Km values, the substrate recognition appears to be the same for both products 1 and 2, while the greatest Vmax value is obtained with compound 2.

The molarity of Tris buffer is an important factor. As can be seen in Figure 5, maximum velocity was obtained at 0.05 M concentration.

Calcium ions have been shown to enhance the activity of certain lipases (17), although their mode of involvement is not definitely known. Calcium could directly modify the behavior of the enzyme (18), but it also modifies the solubilization of the free fatty acid formed during hydrolysis (1). The same has been proposed for the effect of bovine serum albumin (BSA). The effects of calcium chloride and BSA were, therefore, taken into account in our method. As can be seen in Figure 5, no noticeable

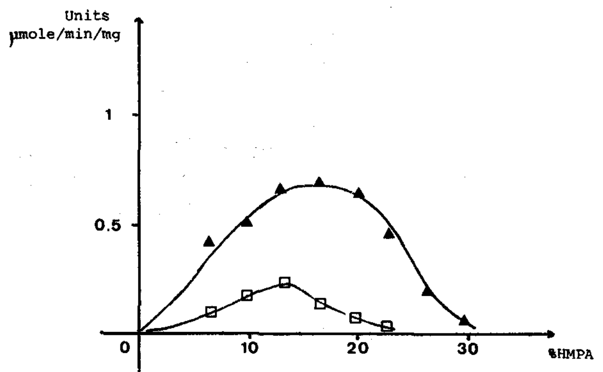

FIG. 2. Effect of hexamethylphosphoric triamide (HMPA) concentration on lipase activity. Experiments were carried out with concentration of compounds 1 (□) and 2 (▲) of 3.5×10^{-4} M and concentration of enzyme of 6×10^{-4} g/l.

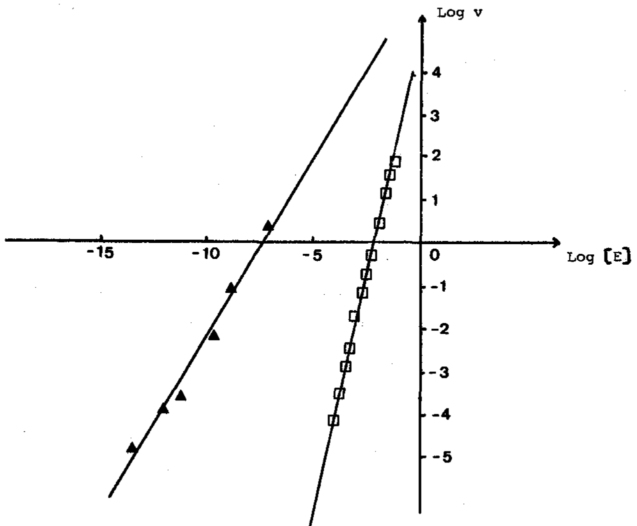

FIG. 3. Effect of increasing enzyme concentration depending on initial velocity with compound 1 (□) and compound 2 (▲). The reaction is linear with enzyme concentration up to 1.2×10^{-4} g/l for compound 1 and 1.6×10^{-4} g/l for compound 2.

TABLE 1

Michaëlis-Menten Constants of Lipase from *Rhizopus arrhizus*[a]

	Substrate	
	Thioglycerol S palmitate (1)	2-Mercaptoethanol S palmitate (2)
K_M mol/l	5.94 10^{-5}	9 10^{-5}
Vmax µmol/min/mg	0.246	0.713
Relative Vmax	1	3

[a]Experiments were carried out using commercial lipase from Gist-Brocades, France.

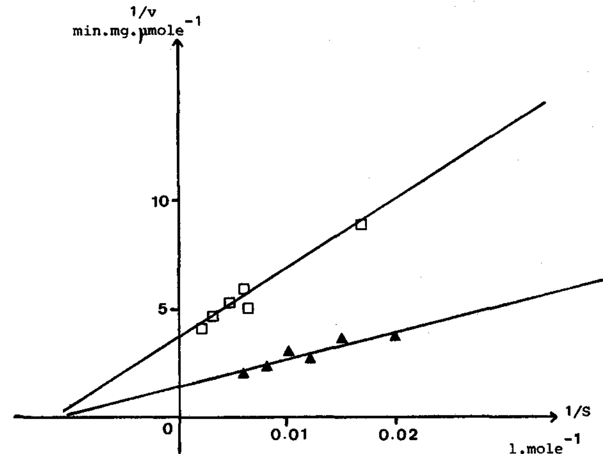

FIG. 4. Michaëlis-Menten treatment of kinetic data for compound 1 (□) and compound 2 (▲).

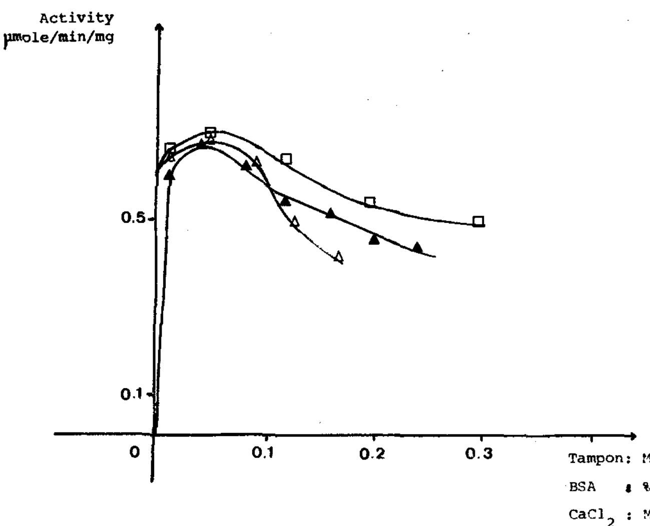

FIG. 5. Effect of buffer molarity (▲), amount of bovine serum albumin (BSA) (□) and calcium chloride molarity (△) on enzyme activity using compound 2.

effect was detected. The observed phenomenon is not sufficiently significant to justify the systematic use of calcium chloride or BSA for improving the sensitivity of the assay.

DISCUSSION

The use of acyl thioesters of mercaptoglycerol in the presence of DTNB has previously been reported (11,14). These authors showed that the results obtained are truly representative of lipase activity. A Japanese patent has recently been registered (19). None of the procedures described, however, is single-step; all require a separation step before lipase activity is measured. With the optically clear medium obtained through using HMPA as the cosolvent of fatty acid thioesters, we eliminated this disadvantage, making it possible to record enzyme activity within 2 min. To compare the sensitivity of this new method with that of the traditional titrimetric assay, we took kinetic measurements of commercial lipase according to the known assay (18). The specific activity that was determined by the titrimetric assay is expressed by a higher number than the one for the improved spectrophotometric method. This may be due to the different types of substrates used (olive oil vs thioesters). However, the spectrophotometric assay is more sensitive. It is possible

TABLE 2

Comparison of the Minimum Amount of Lipase Detected by Different Methods

	Substrate		
	Olive oil	Compound 1	Compound 2
Vmax units/mg	30	0.246	0.713
Minimum amount of lipase (mg/ml)	1	0.075	0.032

to detect lipase activity at levels 10 to 30 times lower when using compounds 1 and 2, respectively (Table 2).

In conclusion, with the improved spectrophotometric method, small amounts of lipase activity can be measured and more assays can be carried out within a short period of time.

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COMMUNICATIONS

Lack of Regression of Preestablished Gallstones in Mice

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Feeding a high cholesterol, high cholic acid diet to mice for 28 days caused increased concentrations of plasma and liver cholesterol and formation of gallstones. Removal of cholesterol and cholic acid from the diet led to normalization of plasma and liver cholesterol within 28 days. Regression of the preestablished gallstones did not occur within 107 days.
Lipids 22, 542–543 (1987).

In various animal models, such as hamsters (1–4), prairie dogs (5) and mice (6,7), selective dietary manipulation causes gallstone formation. In hamsters, gallstones previously induced by feeding a lipid-free diet containing sucrose and casein are solubilized when casein is replaced by soybean protein (3). Gallstone dissolution also occurred when part of the sucrose component was replaced by pectin (4). In prairie dogs, dietary cholesterol–induced gallstones were found to disappear after removal of cholesterol from the diet (5). It thus appears that in these animal models regression of preestablished gallstones will occur after proper dietary change. In the studies mentioned (3–5), regression was seen within seven weeks after institution of the litholytic diet.

In a study in which a high cholesterol, high cholic acid diet was fed to seven inbred strains of mice (8), we fortuitously found the C57BL/U strain to be the only one that developed gallstones. The objective of the present study was to see whether preestablished gallstones in these inbred mice disappear when cholesterol and cholic acid are removed from the diet.

MATERIALS AND METHODS

Female and male mice, aged 7 to 38 weeks, of the C57BL/U strain were used. The strain is fully inbred; it is maintained at the Department of Laboratory Animal Science, Utrecht, The Netherlands. The mice were kept in wire-topped, polycarbonate cages (2 to 14 animals per cage) with a layer of sawdust as bedding. The cages were located in a room with controlled lighting (light, 06.00–20.00 hr; dark, 20.00–06.00 hr), constant temperature (18–20 C) and constant relative humidity (55–65%).

All mice had been fed a commercial pelleted mouse diet (RMH-B®, Hope Farms, Woerden, The Netherlands). On day 0 of the experiment, the mice of each sex were divided into a control and a test group with similar age distributions. The control mice (24 males, 37 females) were fed the commercial diet. The test groups (37 males, 23 females) were fed a pelleted lithogenic diet consisting of the commercial diet (92.5%, w/w), olive oil (5%), cholesterol (2%) and cholic acid (0.5%). Food and water were provided ad libitum.

On days 22, 23, and 27, mice were examined by veterinarians to assess possible discomfort; results have been published elsewhere (9). On day 28, 12 mice of each sex and diet group were chosen randomly for blood sampling and gallbladder examination. Blood samples were taken in the nonfasting state (between 09.00 and 13.00 hr) by orbital puncture into heparinized tubes under light diethyl ether anesthesia. Then, the anesthetized animals were killed by cervical dislocation, and the livers were removed. The gallbladder was examined visually for the presence of gallstones.

From day 28 on, the remaining control mice continued to receive the control diet for another 107 days. On day 135, they underwent the procedure described above. Mice that had been fed the lithogenic diet were transferred to the control diet. Six or seven animals were chosen for autopsy at days 56, 70, 93 and 135 of the experiment.

Plasma total cholesterol was determined enzymatically using a kit (Monotest®) supplied by Boehringer Mannheim GmbH (Mannheim, FRG). Liver cholesterol was extracted and analyzed according to the procedure of Abell et al. (10).

RESULTS AND DISCUSSION

Table 1 shows that feeding the lithogenic diet for 28 days caused increases in plasma cholesterol concentrations of 44 and 82% in male and female mice, respectively. Liver cholesterol had increased dramatically. There was a 20-fold increase in both males and females. The lithogenic diet increased liver weight. Hepatomegaly, expressed as percentage increase in liver wet weight, was about 50%. The enlarged livers showed a grey-white appearance.

After 28 days, the lithogenic diet significantly induced the formation of gallstones in male and female mice, the incidences being 11 and 8 out of 12 animals, respectively (Table 1). The gallstones within the gallbladder could be observed by the naked eye; they were present as spherical aggregates with diameters ranging between 0.1 and 0.8 mm. The gallbladders of the mice fed the lithogenic diet were markedly expanded by the accumulation of bile fluid. The stones moved freely within the bladder. Ebihara and Kiriyama (7), also using a high cholesterol, high cholic acid diet, reported similar observations in male ICR mice, and the gallstones were found to be typical cholesterol gallstones.

Table 1 also shows data of mice placed on the control diet after 28 days on the lithogenic diet. After another 28 days (day 56 of the experiment) on the control diet, plasma cholesterol had fallen toward control values in female mice, but was still somewhat increased (by about 10%) in the males. This increase in the male mice persisted throughout the experiment; it was not due to a time effect because plasma cholesterol concentrations remained constant in the mice fed the control diet during the entire experiment.

Within 28 days on the control diet (day 56) following the lithogenic diet, liver cholesterol concentrations had already dropped to values seen in animals fed the control diet all the time. Liver weight probably returned to control values, although at certain days (70 and 93), liver

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TABLE 1

Influence of Control and Lithogenic Diet on Plasma and Liver Cholesterol and Incidence of Gallstones

Day	Sex	n	Body wt (g)	Plasma cholesterol (mmol/l)	Liver wt (g)	Liver cholesterol (μmol/g)	Gallstone incidence
Control diet							
28	M	12	26.5 ± 1.1	1.99 ± 0.33	1.6 ± 0.1	6.8 ± 0.6	0/12
28	F	12	22.0 ± 1.3	2.30 ± 0.33	1.3 ± 0.1	8.3 ± 1.1	0/12
135	M	12	28.4 ± 2.2	1.94 ± 0.21	1.9 ± 0.2	7.6 ± 0.6	0/12
135	F	11	22.7 ± 1.6	2.14 ± 0.20	1.4 ± 0.2	8.9 ± 0.7	0/11
Lithogenic diet							
28	M	12	26.8 ± 0.9	2.87 ± 0.44	2.6 ± 0.2	144.4 ± 24.1	11/12
28	F	12	19.6 ± 4.5	4.18 ± 1.77	2.2 ± 0.3	179.0 ± 33.5	8/12
Lithogenic diet → Control diet							
56	M	6	28.8 ± 1.6	2.11 ± 0.40	1.8 ± 0.3	7.0 ± 0.5	6/6
56	F	6	23.6 ± 1.4	2.26 ± 0.20	1.6 ± 0.2	7.9 ± 2.8	3/6
70	M	6	31.2 ± 2.4	2.18 ± 0.17	2.1 ± 0.3	7.2 ± 1.1	6/6
70	F	6	24.4 ± 2.3	2.49 ± 0.22	1.4 ± 0.4	8.3 ± 0.5	6/6
93	M	6	28.6 ± 1.8	2.35 ± 0.18	2.1 ± 0.3	6.5 ± 1.1	4/6
93	F	6	22.8 ± 5.3	2.26 ± 0.19	1.4 ± 0.4	9.5 ± 0.7	6/6
135	M	6	27.9 ± 1.7	2.21 ± 0.11	1.9 ± 0.1	7.5 ± 0.9	4/6
135	F	7	25.0 ± 3.1	2.23 ± 0.29	1.5 ± 0.2	8.9 ± 1.1	6/7

Results expressed as means ± SD. On day 0, mice were divided into two groups per gender. Control mice (24 males, 23 females) remained on the commercial (control) diet; the other groups (37 males, 37 females) were transferred to the lithogenic diet. On day 28, some mice on both control and lithogenic diets were autopsied. The remaining mice were either maintained longer on the control diet or transferred from the lithogenic to the control diet for another period of up to 107 days. On day 23, one male mouse in the group on the lithogenic diet died.

weight may still have been somewhat increased in the males.

Table 1 shows that despite normalization of plasma and liver cholesterol concentrations, there was no regression of gallstones. Even after a period of 107 days (day 135) on the control diet the animals invariably displayed gallstones. Within 28 days (day 56) on the control diet, gallbladders were no longer expanded, and gallstones were packed together in the bladder.

It is unfortunate that we do not have information about the composition and concentration of biliary lipids. In any event, it is very difficult to obtain samples of bile fluid from mice fed the control diet, irrespective of whether these animals bear gallstones or not, because the total volume of bile fluid is extremely small. The inbred mice used in this study show a relatively small serum cholesterol response to a high cholesterol diet (8). Six inbred strains (8) that were more susceptible to cholesterol feeding were found not to develop gallstones. Possibly, susceptibility to cholesterol-induced hypercholesterolemia and gallstone formation are inversely related to some extent.

It is clear from this study using mice that gallstones previously induced by feeding high amounts of cholesterol and cholic acid do not disappear, at least not within 107 days, when cholesterol and cholic acid are removed from the diet. In contrast, earlier work using prairie dogs (5) and hamsters (3,4) has suggested that preestablished gallstones may dissolve within seven weeks after feeding a litholytic diet. This discrepancy may be related to the use of different animal species and/or different lithogenic and litholytic diets.

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Purification of High Affinity Fatty Acid Receptors in Rat Myocardial Sarcolemmal Membranes

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High affinity receptors for fatty acid were purified from rat cardiac sarcolemmal membrane using gel filtration, DEAE-cellulose chromatography and affinity chromatography. The purified protein was homogeneous on polyacrylamide gel electrophoresis with the molecular weight of 60 kDa. Binding studies revealed the presence of a single class of high affinity binding sites with an apparent dissociation constant of 1.0 μM and a maximal binding capacity of 12.1 pmol/μg protein.
Lipids 22, 544–546 (1987).

Cytosolic proteins, which are capable of binding fatty acids, have been identified in various tissues, e.g., intestinal mucosa, liver and myocardium (1–3); these are called fatty acid binding proteins (FABP). These cytosolic proteins are considered to aid fatty acid distribution to various pools and pathways, affecting many enzyme reactions.

Fatty acid incorporation through the membrane phospholipid bilayer has been considered to be a simple diffusion process (4,5). However, *Escherichia coli* is reported to have a transport system for fatty acids (6), and kinetic studies suggested that at least a portion of the cellular fatty acid uptake is carrier-mediated (7). Furthermore, membrane receptors responsible for fatty acid binding are isolated in rat liver plasma membrane (8) and kidney basolateral membrane (9). These studies indicate that fatty acid transport across membrane is at least partly a carrier-mediated process. We report here data that have led us to conclude that rat heart sarcolemmal membranes contain a single class of high affinity receptors for fatty acids, which was purified to apparent homogeneity.

MATERIALS AND METHODS

Materials. [^{14}C]Palmitic acid (58 mCi/mmol) was obtained from Amersham International (Arlington Heights, IL); biochemicals were from Sigma (St. Louis, MO); salts and solvents were from Wako (Tokyo, Japan); Sephadex G-75 and Sepharose 4B were from Pharmacia (Uppsala, Sweden); DE-52 was from Whatman (Springfield, U.K.); Lipidex-1000 was from Packard (Downers, Grove, IL) and Bio-Beads SM-2 were from Bio-Rad (Richmond, CA).

Preparation of sarcolemmal vesicles. Rats were anesthetized with ether and hearts were excised and cooled in ice-cold saline; the tissue samples were finely minced with scissors and homogenized in sucrose (0.3 M) containing $MgSO_4$ (5 mM) buffered with imidazole-HCl (10 mM, pH 7.0). Sarcolemmal vesicles were purified by a method analogous to the one described by Reeves and Sutko (10). The yield of the membrane vesicles, starting with ventricular tissue from 20 rats, was 20–30 mg of protein. They showed a sevenfold increase over the crude homogenate in the activity of ouabain-sensitive (Na^+ + K^+)-stimulated, Mg^{2+} dependent ATPase (E.C. 3.6.1.3), widely considered to be the marker for sarcolemmal membranes, and a very low enrichment of cytochrome c oxidase and Ca^{2+}ATPase, the markers for mitochondria and sarcoplasmic reticulum.

Binding of [^{14}C]fatty acids. [^{14}C]Fatty acid binding was determined by the method of Glatz and Veerkamp (11). Unless otherwise stated, incubation mixtures consisted of 1 μM [^{14}C]palmitate, Pi/KCl (0.1 M KCl/0.05 M potassium phosphate, pH 7.5) and protein in a total volume of 0.45 ml. Nonspecific binding was evaluated in parallel incubations of FABP denatured by prior incubations for 4 hr at 70 C. To obtain specific binding, this value was always subtracted from the initial value. All incubations were carried out at 37 C for 15 min. The reactions were stopped by adding 0.05 ml of Lipidex suspension (0.1 g dry Lipidex/ml 0.05 M potassium phosphate), which adsorbs unbound fatty acids. Following vigorous mixing and centrifugation, 0.4 ml of the supernatant was withdrawn and the radioactivity was measured. Before precise FABP activity assay, detergent was removed by Bio-Beads SM-2.

Purification of FABP. Sarcolemmal vesicles were solubilized with 1% (v/v) Triton X-100. After centrifugation at 100,000 × g for 60 min, the Triton-extracted supernatant was delipidated on a Lipidex column (2.6 × 16 cm) (10) and gel-filtered through a Sephadex G-75 column (2.6 × 70 cm) with buffer A (Pi/KCl/0.25% Triton X-100). FABP-rich fractions were pooled and applied to a DE-52 column (2.1 × 15 cm) equilibrated with buffer A. After being washed with buffer A, the column was developed with a linear gradient of 0.1 M—0.6 M KCl in buffer A (200 ml). FABP-rich fractions were pooled and applied to a Bio-Beads SM-2 column (1.5 × 5 cm) equilibrated with Pi/KCl to remove Triton X-100. The sample was then applied to an oleate-Sepharose 4B column (1.5 × 6 cm) prepared by the method of Peters et al. (12) and equilibrated with Pi/KCl. Then the column was washed with the same buffer; no FABP activity was eluted. The column was then developed with Pi/KCl containing 0.5% Triton X-100. Detergent was removed by Bio-Beads SM-2, and the FABP-rich samples were pooled and concentrated by ultrafiltration (UK-10, Toyo, Tokyo, Japan) and used as final FABP.

Other methods. Protein was determined by the method of Sedmak and Grossberg (13), and polyacrylamide gel electrophoresis was carried out using the system of Maizel (14).

RESULTS

Purification of FABP. The results of purification are summarized in Table 1. Of the total protein in the membrane fractions, ca. 89% was recovered after solubilization. The final recovery of FABP was 17.2% of the total activity, and its specific activity was 10.4 pmol/μg protein. SDS-polyacrylamide gel electrophoresis patterns from the

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Abbreviation: FABP, fatty acid binding protein.

TABLE 1

Purification of FABP from Rat Myocardial Sarcolemmal Membrane

Steps	Total protein (mg)	Total activity (pmol)	Specific activity (pmol/μg protein)[a]	Yield (%)	Purification (-fold)
Membrane	27.0	6750	0.25	100	1
Triton extract	13.1	6008	0.46	89	1.8
Sephadex G-75	2.07	3578	1.73	53.0	6.9
DE-52 chromatography	0.69	2093	3.05	31.8	12.2
Oleate-AH Sepharose 4B	0.11	1161	10.4	17.2	41.6

[a]Measured with 1 μM [^{14}C]palmitate at 37 C.

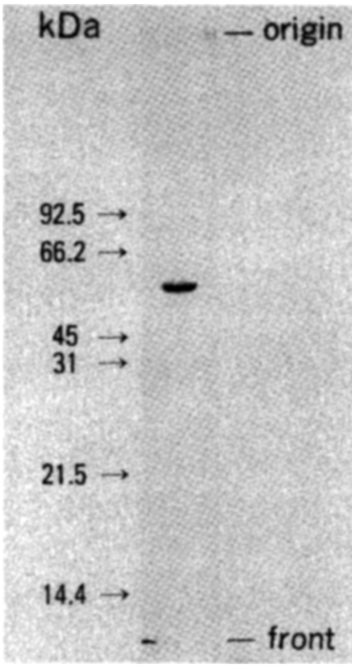

FIG. 1. Electrophoresis of 10 μg of FABP on 0.1% SDS-10% polyacrylamide gel. Proteins of known subunit molecular weight used to calibrate in the gel system included lysozyme (14.4 kDa), soybean trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa) and phosphorylase B (92.5 kDa).

solubilized proteins revealed multiple bands. In contrast, the final eluate from the Sepharose 4B column contained, after reduction, only a single band of estimated molecular weight of 60 kDa (Fig. 1). Purified FABP was stored at −70 C and was stable for up to 4 wk.

Amphiphilicity of FABP. On gel filtration through Sephadex G-75, purified FABP was eluted in a single peak in the presence of Triton X-100, but in several peaks scattered in the absence of the detergent (data not shown), indicated purified FABP forms aggregates in the absence of the detergent. On polyacrylamide gel electrophoresis, purified FABP did not enter the gel in the absence of 0.25% Triton X-100 containing electrode buffer (data not shown). Purified FABP was completely adsorbed to the oleate–Sepharose 4B column in the absence of the detergent, and eluted with 0.5% Triton containing buffer. These results indicate the amphiphilic nature of the purified FABP.

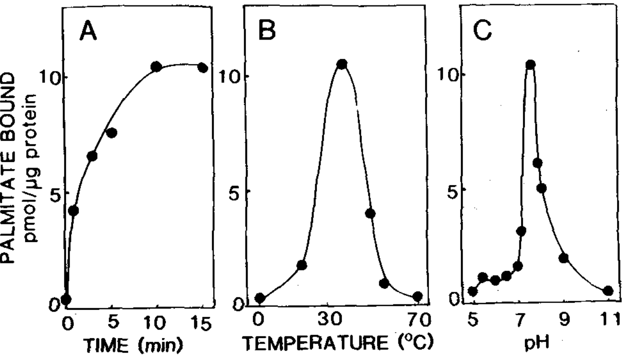

FIG. 2. Time-(A), temperature-(B) and pH-(C) dependence of FABP activity. FABP activity was measured with modifications given below. (A) Incubation for varying time at 37 C with 5 μg FABP (pH 7.6). (B) Incubation at varying temperature for 15 min with 5 μg FABP (pH 7.6). (C) Incubation at varying pH and 37 C for 15 min with 5 μg FABP. Identical pH was observed in potassium- and sodium-phosphate buffers.

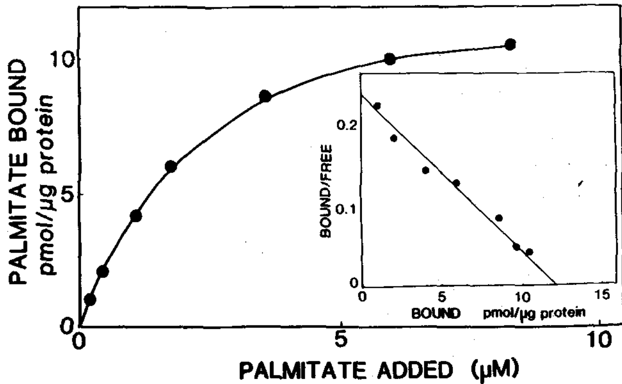

FIG. 3. Binding of palmitate by FABP. Results of a representative experiment is shown. Purified FABP samples (8 μg) were incubated with various concentrations (0.2–8.4 μM) of [^{14}C]palmitate; n = 7. Inset: Scatchard analysis of the binding of palmitate by FABP. The ratio of bound to free [^{14}C]palmitate against the concentration of bound [^{14}C]palmitate is shown. Kd = 1.0 μM, Bmax = 12.1 pmol/μg protein.

Fatty acid binding properties. Several characteristics of the fatty acid binding capacity were investigated. FABP showed rapid and complete binding until 10 min of incubation and optimal binding activity at 37 C and pH 7.6 (Fig. 2). The binding was linear in the range of

TABLE 2

Inhibition by Unlabeled Ligands for [^{14}C]Palmitate Binding to FABP

Inhibitor	Inhibition (%)
Palmitate	25.5
Stearate	14.8
Oleate	20.0
Arachidonate	19.3
Palmitoyl CoA	30.3
CoA	3.5
Cholesterol	2.1
Phosphatidylcholine	3.8

Purified FABP (5 μg) was preincubated for 15 min at 37 C with unlabeled ligands (5 μM in all cases) prior to the addition of [^{14}C]palmitate (1 μM). Inhibition (%) is the ratio to the binding activity without unlabeled ligand.

5-50 μg of the protein. Equilibrium binding of [^{14}C]palmitate to FABP was saturable, and Scatchard analysis revealed the presence of a single class of high affinity binding sites for FABP (Fig. 3). An apparent dissociation constant (Kd) of 1.0 μM and a maximal binding capacity of 12.1 pmol/μg protein were determined. Thus, the purified FABP showed a high affinity for palmitate. The ability of various unlabeled fatty acids, their CoA derivatives, cholesterol and phosphatidylcholine to displace [^{14}C]palmitate from FABP was studied (Table 2). FABP showed a high affinity for palmitoyl CoA but not for free CoA. Various fatty acids showed considerable inhibition, but cholesterol or phosphatidylcholine showed no inhibition at all.

DISCUSSION

These studies demonstrate that rat sarcolemmal membrane has a single protein with a high affinity for fatty acid, which has a molecular weight of 60 kDa and a single class of high affinity binding sites. Based on its molecular weight and the binding capacity, this protein is clearly distinguished from the protein responsible for fatty acid binding shown in rat liver plasma membranes (8), which has a molecular weight of 40 kDa and binding capacity of 3.2 pmol/μg membrane protein for oleate, or the recently reported partially purified FABP in *E. coli* membranes, which has a molecular weight of 26.5 kDa and binding capacity of 3.0 pmol/μg membrane protein for palmitate (15). The results show also that this protein is different from the cytosolic cardiac FABP (16,17), which is a soluble protein with a molecular weight of ca. 14 kDa and large binding capacity for various fatty acids. The affinity of this sarcolemmal FABP for fatty acids has many properties expected of physiologically important binding proteins. The existence of an apparent saturable heat-sensitive binding site with a high affinity for fatty acids is compatible with the appealing hypothesis that fatty acids cross the sarcolemmal membrane by a membrane-associated carrier-mediated transport system and then bind to the soluble cytosolic cardiac FABP previously described. Further studies will be required to determine the role of sarcolemmal FABP in the regulation of myocardial lipid metabolism.

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In Vivo Incorporation of Labeled Fatty Acids in Rat Liver Lipids After Oral Administration

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Striking differences were found in the compartmentalization of fatty acids into liver lipid fractions. The saturated fatty acids—lauric, myristic, palmitic and stearic—were incorporated into phosphoglycerides at faster rates with increasing chain lengths, while triglyceride incorporation was almost uniform. The degree of incorporation of the unsaturated fatty acids into phosphoglycerides (structural) compared to triglyceride (storage and energy) was the converse of their oxidation rates. The incorporation of oleic, linoleic and α-linolenic acids was mainly into triglyceride, whereas dihomo-γ-linolenic acid and arachidonic acid were preferentially incorporated into phosphoglycerides. The data suggest that distribution of each fatty acid is different depending on its destination for structural or energy function.
Lipids 22, 553-558 (1987).

Investigations on comparative metabolism of fatty acids in whole body systems (1,2), perfused organs (3) and myocardial preparations (4,5) indicate that cellular uptake and oxidation of fatty acids varied with degree of unsaturation and chain length. Studies carried out in this laboratory have shown that the different fatty acids are oxidized at different rates in weanling rats. Since part of the nonoxidized fatty acids taken up from the circulation is converted to tissue structural lipids (6,7), it is important to know how the fatty acids are distributed in the different lipid fractions.

Animal membrane lipids consist basically of cholesterol and phosphoglycerides, which are the determinants of membrane fluidity (8). The contribution of the phosphoglycerides to the fluidity of membrane depends on the balance between individual phosphoglycerides and the nature of the fatty acyl groups. The fatty acyl-CoA structure (length, unsaturation, double bond position, configuration of bond lengths) plays a significant role in governing the properties of membranes (9,10), lipoprotein complex formation (11) and activities of enzymes. The neutral lipids store fatty acids for energy, but a proportion may also be transferred to structural pools. Sinclair (12) showed that in suckling rats aged 1–15 days, about 25% of the liver triglycerides consists of long chain polyunsaturated fatty acids, compared to less than 8% in adult rats. However, a higher proportion of arachidonic and docosahexaenoic acids was incorporated into liver phosphoglycerides than into triglycerides in suckling rat pups.

The study reported here examined further the incorporation and compartmentalization of fatty acids into individual phosphoglycerides and neutral lipids, after the main oxidative phase, 24 hr following oral administration of labeled lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1n-9), α-linolenic (18:3-n-3), linoleic (18:2n-6), γ-linolenic (18:3n-6), dihomo-γ-linolenic (20:3n-6) and arachidonic acids (20:4n-6).

Abbreviations: CPG, choline phosphoglycerides; EPG, ethanolamine phosphoglycerides; SPG, serine phosphoglycerides; IPG, inositol phosphoglycerides; RSA, relative specific activity; MCT, medium chain triglycerides.

MATERIALS AND METHODS

Animals. For the early part of the experiment, Sprague-Dawley female rats of the CFY strain were bred in the Institute of Zoology; after weaning at 21 days, they were used for the metabolic experiments. However, at a later date, weanling rats of the same genetic strain were obtained from Benton and Kingman (Yorkshire, U.K.).

The rats were kept under controlled conditions with a 12-hr light-dark cycle, a temperature range of 19–23 C and relative humidity of ca. 55%. Animals were fed a special low calorie RM3 breeding diet obtained from the Special Diet Service (Witham, Essex, U.K.) containing 3.2% fatty acids, whose composition was 12:0 (0.05%), 14:0 (0.19%), 14:1 (0.1%), 16:0 (0.3%), 16:1 (0.1%), 18:0 (0.1%), 18:1 (1.0%), 18:2n-6 (1.0%), 18:3n-3 (0.16%) and 20:4n-6 (0.16%).

Weaned rats, 23–26 days old and weighing 60–80 g, were used in these experiments. All experiments within a series were performed at the same time of day to avoid diurnal variations. Animals were kept in the metabolic chamber for 24 hr and had free access to food and water. Labeled [1-^{14}C]lauric acid (26 mCi/mmol), [1-^{14}C]palmitic acid (58 mCi/mmol), [1-^{14}C]stearic acid (60 mCi/mmol), [1-^{14}C]oleic acid (57 mCi/mmol), [1-^{14}C] linoleic acid (57 mCi/mmol), [1-^{14}C]α-linolenic acid (50 mCi/mmol) and [1-^{14}C]arachidonic acid (50 mCi/mmol) were purchased from Amersham International Ltd. (Amersham, Bucks, U.K.). [1-^{14}C]Myristic acid (54.5 mCi/mmol) was obtained from Fluorochem Ltd. (Gosport, U.K.). [1-^{14}C]γ-Linolenic acid (55 mCi/mmol) and [1-^{14}C]dihomo-γ-linolenic acid (55 mCi/mmol) were obtained from Roche Product Ltds. (Hert, U.K.). All were judged 98% pure by radio gas liquid chromatography except linoleic (96%) and dihomo-γ-linolenic (99%) acids. Radiolabeled fatty acid (6 μCi) was introduced into a vial containing 0.2 ml olive oil, and the solvent was evaporated under a stream of N_2. The oil and isotope mixture was then administered orally to the rat through a Jackson catheter (Arnold, Veterinary Product Ltd., Reading, U.K.).

Preparation of the metabolic chamber. After oral administration of the labeled fatty acid, the rat was immediately placed in the "metabolic chamber" (Jencon Ltd., Berk, U.K.). A current of dry carbon dioxide–free air was pumped through the metabolic chamber by a Hyflo Technical Model C pump (Scientific Suppliers, London), carrying with it the expired $^{14}CO_2$ from the rat. The expired $^{14}CO_2$ was dried by being passed through concentrated sulphuric acid and trapped in two collection columns containing a mixture of methoxyethanolamine/ethanolamine (2:1, v/v), 500 ml in the first column and 300 ml in the second column.

In vivo recovery of $^{14}CO_2$. From the first collection

TABLE 1

Percentage of Labeled Fatty Acid Expired as $^{14}CO_2$ in Rats 24 Hours After Oral Administration[a]

Time (hr)	[^{14}C] Fatty acids									
	12:0	14:0	16:0	18:0	18:1	18:2n-6	18:3n-6	18:3n-3	20:3n-6	20:n-6
1	9.9±3.9 (n=9)	3.1±1.2 (n=8)	0.5±0.1 (n=8)	0.3±0.2 (n=8)	4.4±1.9 (n=5)	8.9±3.7 (n=5)	5.0±1.0 (n=8)	2.9±1.8 (n=8)	1.0±0.5 (n=8)	0.8±0.4 (n=7)
3	20.8±6.1 (n=6)	15.3±4.2 (n=4)	6.1±2.0 (n=6)	5.6±2.8 (n=4)	37.0±3.1 (n=5)	13.0±4.9 (n=5)	5.5±1.0 (n=8)	21.0±8.9 (n=4)	2.0±0.5 (n=8)	2.6±1.1 (n=3)
7	46.0±6.7 (n=6)	30.0±6.7 (n=4)	15.0±3.9 (n=6)	17.0±1.8 (n=4)	49.0±1.9 (n=5)	28.0±5.8 (n=5)	13.0±1.5 (n=8)	43.0±1.5 (n=4)	5.0±1.5 (n=8)	5.7±1.3 (n=3)
24	63.0±7.7 (n=9)	40.0±7.3 (n=8)	32.0±3.2 (n=8)	25.0±2.8 (n=8)	57.0±2.1 (n=8)	48.0±2.8 (n=9)	27.0±3.0 (n=8)	64.0±2.9 (n=8)	14.0±1.5 (n=8)	14.0±7.3 (n=7)

Results are expressed as mean ± SEM.
[a]Expressed as percentage of the administered dose.

column, 10-ml aliquots of trapping agent were removed at 1, 3, 7 and 24 hr. At 24 hr, 10 ml was also removed from the second column to account for any overflow of labeled CO_2 from the first column. From each sample, four 2-ml aliquots were removed and added to scintillation vials containing 10 ml scintillation fluid (0.81 g Scintimix 2, [25% PPO, 5% dimethyl POPOP], dissolved in a 180-ml mixture of toluene/methoxyethanolamine/ethanolamine [100:70:10, v/v/v]) and counted.

Lipid extraction. After 24 hr, the rats were killed by decapitation, and the livers were removed, washed in ice-cold saline, blotted dry, and then weighed. Lipids were extracted using the method of Folch et al. (13). The liver was homogenized with a 20-fold volume of chloroform/methanol (2:1, v/v) containing 10 mg of butylated hydroxytoluene (BHT)/l as an antioxidant, which was then flushed with nitrogen and left to extract overnight at 4 C. The homogenate was filtered through Whatman No. 1 filter paper, and the residue was washed with an additional 10 ml of chloroform/methanol (2:1, v/v). The filtrate was transferred to separation funnel and diluted to 100 ml with chloroform/methanol (2:1, v/v), mixed with 20 ml of 0.85% sodium chloride (v/v), shaken and left to partition overnight at 4 C. The lower organic phase was transferred to a round-bottom flask and evaporated with N_2 in a Rotavap-R (Buchi, Orme Scientific, Middleton, Manchester, U.K.), under reduced pressure at 37 C. The lipid extract was redissolved in 20 ml of chloroform, flushed with N_2 and stored at 4 C. Two 2-ml aliquots were removed from the lipid extract and transferred to scintillation vials. The solvent was evaporated under N_2, 10 ml scintillation fluid (Unisolve 1, Koch-Light Laboratories, Buckinghamshire, U.K.) was added and the samples were counted.

Thin layer chromatography. The lipid classes were separated by thin layer chromatography using Silica Gel G for neutral lipid and Silica Gel H for phosphoglyceride separation. A 2.5-ml aliquot of liver lipid extract containing ca. 10 mg of lipid was evaporated to dryness under N_2, redissolved in a small volume of chloroform (10 μl) and applied to the plate as a narrow band using a capillary tube.

The solvents used for phosphoglyceride separation were chloroform/methanol/n-propanol/methylacetate/0.25% potassium chloride (25:10:25:25:9, v/v/v/v/v). The solvents used for the separation of neutral lipids were petroleum spirit/diethyl ether/glacial acetic acid/methanol (85:15:2.5:1, v/v/v/v). Samples were always run with known standards. The phosphoglyceride fractions were identified by placing the plate in a tank of iodine vapor in which they appeared as brown bands. The neutral lipids plate was sprayed with a methanolic solution of dichlorofluorscein (0.2%, v/v), and the lipid fraction was identified under UV light. The separated lipid classes were then scraped into scintillation vials. Ten ml of scintillation fluid (Unisolve 1) was added, and the samples were counted.

Radioactivity was measured by scintillation counting using a Model SL30 liquid scintillation counter (Intertechnique, Plaisir, France). The counting efficiency for the lipid and trapping agent was normally about 85% and was determined by addition of an internal standard [1-^{14}C]hexadecane and by external standard channel ratio mode. Using this method, the recovery of radioactivity from the plates was greater than 90% for each fatty acid.

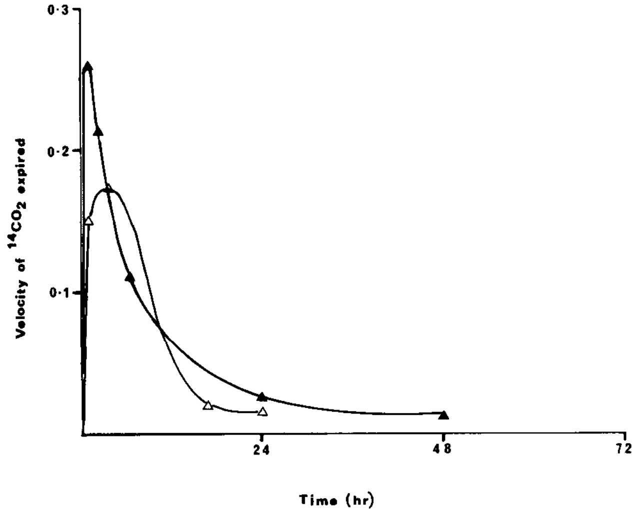

FIG. 1. Velocity time curves for the oxidation of [^{14}C]linoleic acid (▲) and [^{14}C]myristic acid (△) were measured by plotting the gradient of the percentage of expired $^{14}CO_2$ curve against time.

RESULTS

Oxidation of labeled fatty acids. The oxidation of the labeled fatty acids was determined from the expired $^{14}CO_2$ over a 24-hr period after oral administration. The results shown in Table 1 are the cumulative amounts of expired $^{14}CO_2$ recovered as a function of time and expressed as a percentage of the administered dose. In the saturated fatty acid group, lauric and myristic acids were oxidized to a greater extent than palmitic and stearic acids ($P < 0.01$). Of the unsaturated fatty acids, α-linolenic acid and oleic acid were both preferentially oxidized over linoleic acid ($P < 0.01$). Desaturation of linoleic acid to γ-linolenic acid resulted in a significant reduction ($P < 0.01$) of oxidation of the latter to 27%. All the C_{18} fatty acids were preferentially oxidized ($P < 0.01$) over the 20 carbon chain length except γ-linolenic acid ($P < 0.05$).

In a plot of the gradient of the percentage of expired CO_2 curve against time, maximum velocity was reached at 6 hr for linoleic acid and myristic acid and fell to a low level by 24 hr. The result (Fig. 1) shows the rate of CO_2 expired per unit of time, indicating most of the oxidation of fatty acids was completed by 24 hr.

Liver phosphoglyceride fraction. The profile of the labeled fatty acids in the four phosphoglyceride classes obtained from the liver is shown in Figure 2. A significant amount of the radioactivity ($P < 0.001$) was found to be associated with choline phosphoglycerides (CPG) and ethanolamine phosphoglycerides (EPG) compared to the serine phosphoglycerides (SPG) and inositol phosphoglycerides (IPG). In the CPG and EPG fractions, the saturated fatty acids were incorporated in this order: stearic acid > palmitic acid > myristic acid > lauric acid. For the unsaturated fatty acids, the order was arachidonic acid > dihomo-γ-linolenic acid > γ-linolenic acid > linoleic acid > α-linolenic acid > oleic acid into phosphoglycerides. Increasing desaturation and elongation of the fatty acids paralleled increasing incorporation into phosphoglycerides and also reflected their oxidation rates. For example, arachidonic acid, which was oxidized the least (13.9% in 24 hr), was incorporated the most into phosphoglycerides, whereas lauric acid, which was oxidized the most (63.0% in 24 hr), was incorporated the least ($P > 0.001$).

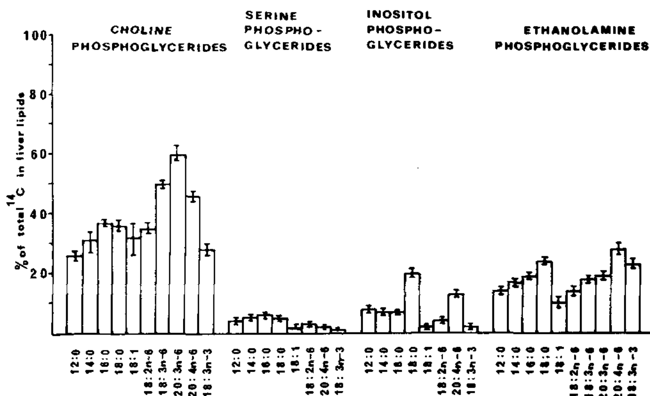

FIG. 2. The in vivo incorporation of labeled fatty acids into rat liver phosphoglycerides 24 hr after oral administration. Phosphoglycerides were separated by thin layer chromatography and radioactivity incorporated into the phosphoglyceride fractions measured by scintillation counting. The results of each fraction are expressed as a percentage of the total activity recovered. Vertical bars give the SEM for a total of 8 tissues analyzed for each fatty acid.

Liver neutral lipids. Figure 3 shows the incorporation of labeled fatty acids into triglycerides and total phosphoglycerides. Oleic acid was most significantly incorporated ($P < 0.001$) into triglyceride (63.4%), compared to its incorporation into total phosphoglycerides (34.0%). Of the n-6 family of fatty acids, linoleic acid was preferentially incorporated into triglycerides compared to its long chain derivatives. By contrast, the saturated fatty acids (C_{12}, C_{14}, C_{16} and C_{18}) showed almost uniform distribution into triglycerides.

Incorporation into liver phosphoglycerides in terms of relative specific activity (RSA). Table 2 compares the incorporation of labeled fatty acids into phosphoglycerides and triglycerides, relative to the actual amounts of phosphoglycerides present in the rat liver (percentage activity recovered divided by percentage distribution in rat liver

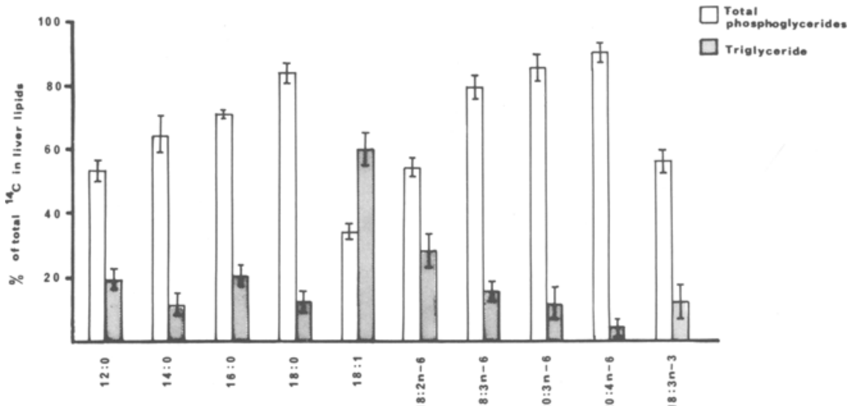

FIG. 3. The in vivo incorporation of labeled fatty acids into rat liver lipids 24 hr after oral administration. Total phosphoglycerides were calculated by adding up the individual phosphoglyceride fractions. Liver triglycerides were obtained by neutral lipid separation on thin layer chromatography. The results are expressed as a percentage of the total activity recovered. Vertical bars give the SEM for a total of 8 tissues analyzed for each fatty acid.

TABLE 2

Relative Specific Activity of Labeled Fatty Acids Incorporated into Rat Liver 24 Hours after Oral Administration[a]

	[¹⁴C] Fatty acids									
Phosphoglycerides[b]	12:0	14:0	16:0	18:0	18:1	18:2n-6	18:3n-6	18:3n-3	20:3n-6	20:4n-6
SPH	0.1 (n=6)	1.4 (n=8)	1.2 (n=10)	0.8 (n=8)	0.8 (n=12)	0.3 (n=7)	—	0.2 (n=8)	—	0.4 (n=8)
CPG	1.2 (n=6)	1.6 (n=8)	1.8 (n=10)	1.7 (n=8)	1.5 (n=12)	1.6 (n=9)	2.3 (n=8)	1.4 (n=8	2.9 (n=8)	2.2 (n=8)
SPG	3.2 (n=6)	5.0 (n=8)	5.2 (n=10)	4.3 (n=8)	1.6 (n=12)	2.8 (n=9)	—	1.4 (n=8)	—	1.5 (n=8)
IPG	2.9 (n=6)	2.6 (n=8)	2.4 (n=10)	6.7 (n=8)	0.6 (n=12)	1.5 (n=9)	—	0.9 (n=8)	—	4.6 (n=8)
EPG	4.7 (n=6)	1.6 (n=8)	1.8 (n=10)	2.3 (n=8)	1.0 (n=12)	1.4 (n=9)	1.8 (n=8)	2.3 (n=8)	1.8 (n=8)	2.7 (n=8)
TG	0.4 (n=6)	0.3 (n=8)	0.5 (n=10)	0.2 (n=8)	1.5 (n=12)	0.8 (n=9)	0.4 (n=8)	0.7 (n=8)	0.3 (n=8)	0.1 (n=8)

[a]Values are expressed as percent activity recovered divided by the percentage distribution of phosphoglycerides and triglyceride present in the rat liver (per g of tissue).

[b]SPH, sphingomyelin; CPG, SPG, IPG and EPG are choline, serine, inositol and ethanolamine phosphoglycerides, respectively; TG, triglyceride.

phosphoglycerides). Although the CPG fraction contained the largest amounts of activity in the phosphoglycerides, the RSA data suggested that the SPG fraction was especially active for the saturated fatty acids, whereas the IPG fraction was selectively rich in arachidonic acid. The RSA for linoleic acid was found to be substantial in the SPG fraction. The RSA for the fatty acid composition in CPG and EPG fractions were similar. There was very little cholesteryl ester in the rat liver lipids; the data are not reported because the counts were too low for confidence.

DISCUSSION

Oxidation of fatty acids. Whether dietary fatty acids are oxidized for energy or used in structural lipids depends on various factors, which may include selective mechanisms, type of fatty acid and the metabolic activity and hormonal and nutritional status of the animal. Studying changes in fatty acid incorporation into lipids is also complicated kinetically in vivo, since labeled fatty acids are subsequently diluted by endogenous fatty acids. However, in our experiments, we were dealing with growing animals in a normal metabolic condition in which comparisons were being made between the behavior of different fatty acids under the same dietary conditions.

The oxidation rates of the labeled administered fatty acids are unlikely to have been influenced by the dietary fatty acids for several reasons: (i) The animals were fed a low fat diet (3.2% of the diet). (ii) The diet contents of myristic (0.2%), α-linolenic (0.2%) and arachidonic acids (0.2%) were similar, but the oxidation rates of the fatty acids were quite different, with [¹⁴C]α-linolenic acid being catabolized at a substantially higher rate than 20:4n-6. (iii) Oleic and linoleic acids both were present in the diet at 1.0%. However, their labeled counterparts showed a significant difference in oxidation rates, with oleic acid being oxidized much faster than linoleic acid. (iv) Palmitic acid was present in the diet in higher amounts (0.3%) than stearic acid (0.1%). Had the fatty acids present in the diet acted to dilute the label, then one would have expected palmitic acid to be diluted and oxidized at a slower rate then stearic acid, but the converse was true, with stearic acid being oxidized at the slowest rate of the saturated fatty acids. The differential metabolism of the fatty acids was due to their inherent properties. There was simply no consistency with the small amounts of fatty acids in the diet and the rates of oxidation. The different rates of fatty acid oxidation were also consistent with the different incorporation into storage (triglycerides) and structural (phosphoglycerides).

The amount of labeled fatty acid excreted in the feces was very low (1% of the administered labeled fatty acids) after 24 hr and serves only as a partial index of fatty acid absorption by the gut. Higher activity may be present in the intestine and colon: however, this was not measured. The activity excreted in the urine was slightly higher (about 3% of the administered dose).

Compartmentalization. The results obtained for the incorporation of fatty acids into rat liver lipids are presented with the realization that a time-course study would have made clear the interpretation of fatty acid compartmentalization. However, given the logistics and the time involved in the analytical procedure and because most of the fatty acids reached their slowest rate of oxidation at 24 hr, we decided to measure the incorporation at this time point.

The data describing the incorporation of fatty acids into liver phosphoglycerides were expressed in percentages. The results show that a strongly selective mechanism exists to direct fatty acids into different lipid pools. Examination of labeled fatty acid profiles in the phosphoglyceride fractions showed that liver CPG and EPG contained much higher percentages of labeled fatty acids than SPG and IPG.

Relative specific activity (RSA). Since the lipid frac-

tions are present in different amounts in the liver, the data representing the distribution of fatty acids describes only how much of an individual fatty acid has been incorporated in a specific direction. But since SPG and IPG are present in only small amounts, they may be taking up a fatty acid more actively and thus have a high activity per unit. This type of information can be obtained by expressing the radioactivity per mole, or RSA.

Saturated fatty acids. The RSA data indicated a high incorporation of activity into the SPG and IPG fraction, especially for the saturated fatty acids. The high RSA in SPG and IPG suggests different turnover rates for the individual phosphoglyerides. The results from this study showed that stearic and palmitic acids were incorporated into SPG in preference to the medium chain triglyceride (MCT) fatty acids. Coots (1), who measured the oxidation of stearic acid, found that a significant amount was incorporated into lymph phosphoglycerides. The preferential incorporation of stearic and palmitic acids into phosphoglycerides (2) might also explain the slower oxidation for the long chain saturated fatty acids.

Polyunsaturated fatty acids. Linoleic acid was found to be preferentially incorporated into triglycerides compared to its long chain derivatives, 20:3n-6 and 20:4n-6, which were incorporated mainly into phosphoglycerides. Sinclair (12) also found that in suckling rats, linoleic acid was incorporated into triglyceride, and much of it was derived from the dam's milk. The high turnover rate for triglyceride (14) may well account for the rapid rate of oxidation for 18:2n-6. α-Linolenic acid was also found to be incorporated into triglycerides and is consistent with the observations that the high oxidation rates of the C_{18} is associated with the triglyceride.

The special place of oleic acid. The results from this study showed that oleic acid was preferentially incorporated into triglycerides. Similar findings have also been reported in the perfused heart (15), where oleic acid was incorporated more into phosphatidic acid, suggesting substrate specificity for diacylglycerol acyltransferase (16). Yan and Sun (17) injected labeled oleic acid intracerebrally and found that it was initially incorporated into triglyceride. However, after 80 min, the activity in phosphoglycerides gradually increased with simultaneous reduction in the triglycerides.

Oleic acid is an important monounsaturated fatty acid in the developing human fetal brain (18). It also accounts for the major fatty acid in human milk (19), ranging from 30 to 40% (20). Both 18:1n-9 and its metabolic product, nervonic acid (24:1n-9), are major fatty acids found in mature myelin, which appears in the nervous system in the later stages of brain development. These observations suggest that oleic acid can perform two different functional roles. It can serve first as an energy source, as indicated by the high oxidation rate, and second as a substrate for cell growth and development.

Compartmentalization of the C_{20} fatty acids. Unlike the C_{18} fatty acids, the C_{20} long chain PUFA showed preferential incorporation into phosphoglycerides. The RSA data showed that IPG was selectively rich in arachidonic acid, indicating a faster rate of uptake and turnover of this phosphoglyceride fraction. The slow oxidation and high rate of incorporation of arachidonic acid into structural lipids also imply a long half-life. Evidently, the turnover rate for the different fatty acids will be different for the different fatty acids.

Hassam and Crawford (21) found that up to a third of the radioactivity recovered from the liver and plasma lipid fractions 24 hr after dosing with labeled dihomo-γ-linolenic acid (DHLA) was still present as DHLA. Therefore, besides being metabolized to arachidonic acid and the 22-carbon chain length derivatives, it is also available for incorporation as DHLA into tissue lipids.

Similar data have been reported for the compartmentalization of linolenic and arachidonic acids and other PUFA in cultured rabbit-aorta smooth muscle cell (22). The CPG fraction incorporated the highest proportion of the fatty acids. These investigators did not calculate specific activity data, but had they done so, it is likely their IPG and SPG incorporation would have been similar to ours. Morisaki and coworkers (22) found no evidence of desaturation of γ-linolenic acid. While eicosapentaenoic acid was elongated to docosapentaenoic acid, it was not desaturated further to docosahexaenoic acid. Their data on a specific cell type are similar to our observation of the differential compartmentalization of the essential fatty acids in rats and human leukocytes (23) and human placenta (24), suggesting that our in vivo data reflect a general phenomenon at a cellular level.

The fundamental observation made in this work and in other studies is that compartmentalization of fatty acids profoundly influences the metabolic direction and use to which a fatty acid is put. The evidence gained for specificity in compartmentalization and, indeed, the converse evidence for use in energy suggests that the individual fatty acids may need to be considered as separate individuals.

It has been popular to use MCT clinically in treating patients recovering from surgery and trauma, and where there is a need for cell repair and growth, in both enteral and parenteral nutrition. However, our data imply that the MCT fatty acids, while being useful for energy, may not be so for cell structural repair, and consequently can only have limited value. By contrast, oleic acid is oxidized as fast as the fatty acids of MCT, but can also be used for phosphoglycerides and for cell structures. It should be possible to use a lipid, such as olive oil (which is high in oleic, palmitic, stearic and linoleic acids), to satisfy the need for energy as well as cell growth and to repair simultaneously.

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Partial Purification and Characterization of Free and Immobilized Lipases from *Mucor miehei*[1]

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An extracellular lipase, a glycoprotein, produced by fermentation with a selected strain of *Mucor miehei* has been partially purified in two forms, A and B. The two forms have a high degree of antigenic identity and have similar pH-activity profiles with tributyroylglycerol as a substrate with optima at pH 7. They differ as follows: A, in contrast to B, requires activation at alkaline pH before analysis; A binds with concanavalin-A more completely than B; the net charges are slightly different at pH 8; and the isoelectric points are different. Our results indicate that the B lipase is formed by partial deglycosylation of the A lipase and that this influences the activity toward emulsions.

In addition, the two enzymes have been immobilized by adsorption. These preparations and the soluble forms were highly specific for primary esters of triacylglycerols (TG); they usually hydrolyzed TG of 12:0, 14:0, 16:0, and 18:1 more rapidly than those of 4:0, 6:0, and 8:0 and 10:0 in mixtures of monoacid TG (4:0 to 18:1); and they were not stereospecific for TG. Immobilization altered the specificity of the preparations somewhat, in that slightly more 14:0 and 16:0 were released.
Lipids 22, 559–565 (1987).

Enzymatic modification of foods and other materials has occurred either spontaneously or deliberately for generations. The relatively recent availability of large quantities of enzyme preparations has broadened the search for industrial applications of enzymes and simultaneously for enzymes with desirable characteristics such as thermostability and certain specificities. For several reasons, including relative ease of production, many of these enzymes are obtained from microbial fermentations. These aspects, with emphasis on lipases, have recently been discussed by Kilara (1).

During our search for microbial lipases with industrial applications, we selected a strain of *M. miehei* for further study. This mold produces an active extracellular lipase (2). Results from preliminary experiments suggested that there were at least two different forms of the enzyme. In this paper, we report the partial purification of two of these soluble forms. These were immobilized, and the four preparations were partially characterized. The purifications and immobilizations were done in Denmark and the specificity studies in Connecticut. Preliminary data on use in industrial applications and some characterization of the lipase have been presented (3,4). We believe that this is among the few reports (5,6) on the characteristics of immobilized lipases and the first on the A and B forms of *M. miehei* lipases.

MATERIALS AND METHODS

Purification: materials and determinations. DE-cellulose 52 was purchased from Whatman Chemical Separation Ltd. (Kent, England); DEAE-Sepharose, Phenyl-Sepharose, concanavalin A (ConA)-Sepharose and Sepharose 4B were obtained from Pharmacia Fine Chemicals (Piscataway, NJ).

Lipase activity was measured with pH-stat essentially as described by Brockman (7) except that 0.5% polyvinylpyrrolidone was used as an emulsion stabilizer. Substrates were tributyroylglycerol (Novo method AF95.1/3-GB) and olive oil (method AF182.2/3-GB). (These designations are given for those who may use the Novo methods; otherwise they are very similar to [7].) Units of activity are 1 LU (lipase unit), the amount of enzyme that liberates 1 μmol of titratable 4:0/min under standard conditions, and 1 NLU (Novo lipase unit), the amount of enzyme that liberates 1 μmol of tritratable fatty acid from olive oil/min under standard conditions. Note that these Novo methods require that the *Mucor* lipase be diluted in alkaline buffer (pH 10.5) prior to analysis. This buffer did not interfere with subsequent analyses. Protein determination was performed according to Lowry et al. (8) with bovine serum albumin as a standard.

Purification of lipase A. (All steps were carried out at 5 C.) The supernatant from the culture broth of a selected strain of *M. miehei*, with mycelia and low molecular weight substances removed, was used for production of a crude powder. (a) Step 1: anion exchange chromatography. A solution (200 ml) of 5% of the crude powder was made up with deionized water, adjusted to pH 7 with NaOH and applied to a column (2.5 × 40 cm) of DE-cellulose 52 previously equilibrated with 0.5 M Tris-HCl buffer (pH 7.0). After the unbound proteins were washed out with the buffer, the bound proteins, including lipase, were eluted with a gradient of decreasing pH (initial buffer pH 7.0 to 0.1 M maleate, pH 3.5). The main lipolytic fraction obtained (pH 5.4) was adjusted to pH 7.0 and concentrated to 1/5 volume by ultrafiltration prior to lyophilization. Ultrafiltration was done with an Amicon UF cell using a membrane with a nominal molecular weight cutoff of 10,000.

(b) Step 2: affinity chromatography. A 250-mg volume of powder from the DE-cellulose chromatography was diluted in 2 ml 0.02 M Tris-HCl buffer, pH 7.4, containing 0.3 M NaCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$, and was applied to a column of conA-Sepharose (1.6 × 20 cm) equilibrated with the buffer. The column was washed with the initial buffer for unbound proteins, and the bound lipase eluted with a gradient of 0 to 0.5 M α-D-methyl mannoside in the buffer (400 ml). The flow rate was 20 ml/hr, and fractions of 5 ml were collected. The pooled

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Abbreviations: TG, triacylglycerol; DG, diacylglycerol; MG, monoacylglycerol; conA, concanavalin A; TLC, thin layer chromatography; GLC, gas liquid chromatography; LU, lipase unit; NLU, Nova lipase unit; pI, isoelectric point; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; IEF, isoelectric focusing; CIE, crossed immunoelectrophoresis; CIAE, crossed immunoaffinity electrophoresis.

TABLE 1

Purification of Lipase A from *Mucor miehei*

Purification steps	Yield, % based on LU values[a]	Protein content (%)	Specific activity			
			LU/mg preparation	LU/mg protein	NLU/mg[b] preparation	NLU/mg protein
Crude powder	100	42	157	374	100	238
DE-cellulose 52 chromatography	77	67	1700	2540	1073	1601
ConA-Sepharose chromatography	57	66	3590	5440	2300	3500

[a]LU (lipase unit) is the amount of enzyme that liberates 1 μmol of 4:0/min (see text).
[b]NLU (Novo lipase unit) is the amount of enzyme that liberates 1 μmol of fatty acid from olive oil/min (see text).

TABLE 2

Purification of Lipase B from *Mucor miehei*[a]

Purification steps	Yield, % based on LU values[b]	Protein content (%)	Specific activity			
			LU/mg preparation	LU/mg protein	NLU/mg[c] preparation	NLU/mg protein
Crude powder	100	42	157	374	100	238
DEAE-Sepharose chromatography	72	80	945	1180	600	750
Phenyl-Sepharose chromatography	32	100	3000	3000	1900	1900

[a]Lipase B is formed by partial deglycosylation of lipase A (see Table 1 and text).
[b]LU (lipase unit) is the amount of enzyme that liberates 1 μmol of 4:0/min (see text).
[c]NLU (Novo lipase unit) is the amount of enzyme that liberates 1 μmol of fatty acid from olive oil/min (see text).

lipase fractions were desalted and concentrated to a 1/10 volume by ultrafiltration prior to lyophilization.

Purification of lipase B. (All steps were carried out at 5 C, except for the pretreatment in step 2.) (a) Step 1: anion exchange chromatography. This was performed as described for lipase A, but with the following modifications: The column (2.5 × 40 cm) was DEAE-Sepharose, the final buffer was 0.1 M glycine-HCl pH 3.0 and the low pH (4.5) of the lipase fraction obtained was not adjusted prior to ultrafiltration. (b) Step 2: hydrophobic interaction chromatography. A solution (50 ml) containing 2% of the DEAE Sepharose purified powder in 0.2 M ammonium acetate, pH 4.7, was stirred for 30 min at 3 C. Sufficient ammonium acetate was then added to bring the concentration to 0.8 M. This solution, cooled to 5 C, was applied to a column (2.5 × 40 cm) of Phenyl-Sepharose equlibrated with 0.8 M ammonium acetate buffer, pH 5.1. The unbound proteins were washed out with the buffer prior to elution of the column with deionized water. The eluted lipase fraction (pH not adjusted) was concentrated to a 1/10 volume by ultrafiltration prior to lyophilization.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This was performed as described in Bio-Rad's manual (9) for the Laemmli system, using Bio-Rad vertical slab gel cell model 220. The separating gel was a gradient of 7–20% polyacrylamide. The mercaptoethanol-treated samples were boiled for 2 min before application to the gel. As reference proteins, the electrophoresis calibration kit for molecular weight determination of low molecular weight proteins from Pharmacia was used.

Isoelectric focusing (IEF). This was performed on LKB's Multiphore apparatus using ready-made polyacrylamide gels as described in LKB's manual (10). The IEF calibration kit from Pharmacia was used for reference proteins.

Crossed immunoelectrophoresis (CIE). CIE was performed as described by Hojby and Axelsen (11) using 0.02 M barbital buffer, pH 8.0. The polyspecific antibodies were produced in rabbits against the crude enzyme powder. Tandem-CIE was performed as described by Kroll (12), using 0.02 M barbital buffer pH 8.0.

Crossed immunoaffinity electrophoresis (CIAE). This was performed by modification of the CIE method as follows: The buffer used was 0.01 M Tris-maleate, pH 7.4. An intermediate gel (1 cm broad) was interposed between the first-dimensional gel and the antibody-containing gel. This intermediate gel contained 25 μl conA-Sepharose/cm^2 on the test plate. The control plate had the conA-Sepharose replaced by Sepharose 4B. Before use, the

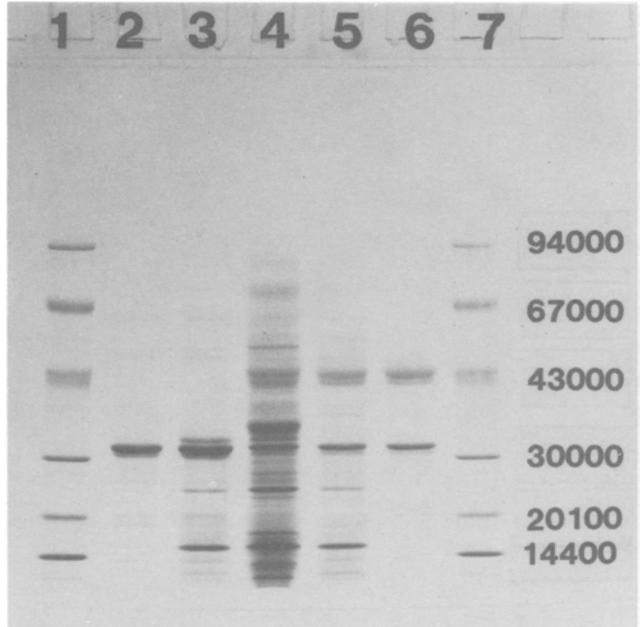

FIG. 1. SDS-PAGE of lipase preparations obtained from the various purification steps. (1) and (7), respectively, 18 μg of the total reference proteins; (2) 25 μg of the conA-Sepharose purified lipase A preparation; (3) 63 μg of the DE-cellulose 52 purified lipase A preparation; (4) 375 μg of the crude powder; (5) 63 μg of the DEAE-Sepharose purified lipase (A/B) preparation; (6) 25 μg of the Phenyl-Sepharose purified lipase B preparation. Proteins were stained with Coomassie Brilliant Blue.

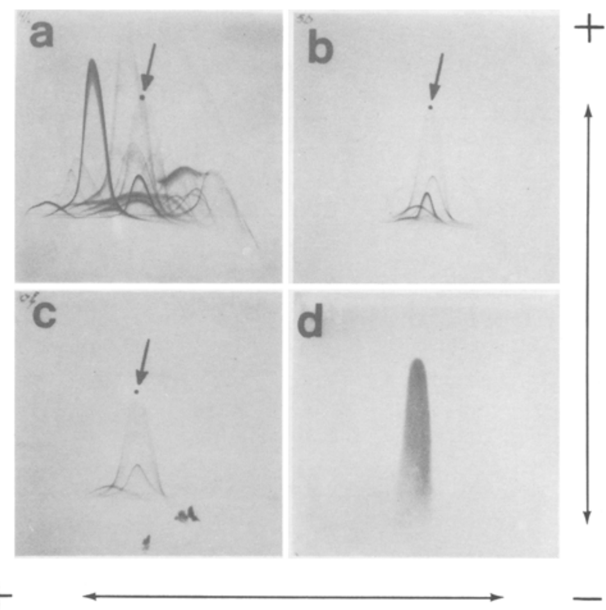

FIG. 2. Crossed immunoelectrophoresis (CIE) of purification steps of lipase A. a, b and c are CIE-stained with Coomassie Brilliant Blue. The arrow indicates the lipase-containing immunoprecipitate, detected as described for d. Part d is the duplicate CIE of b but with tributyroylglycerol overlayer, where the lipase peak shows as a clearing zone. The antigens applied were as follows: a, 100 μg of crude powder; b and d, respectively, 10 μg of DE-cellulose 52 purified lipase A preparation; c, 5 μg of conA-Sepharose purified lipase A preparation. 500 μl of the polyspecific antibodies were applied on each CIE. In the figures, cleared zones appear as dark areas because a dark background was used during photography. Otherwise, the zones cannot be visualized.

conA-Sepharose as well as the Sepharose 4B were equilibrated with the buffer containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$ and then were added as a 50% slurry to the agarose gel.

Overlayer technique for detection of lipase activity. Bands and precipitates in IEF and CIE/CIAE, which contained lipases, were detected as follows: Immediately after the electrophoretic run, the plate was incubated with ~1.5 mm overlayer gel (1% agarose) containing a tributyroylglycerol emulsion at a final concentration of 1% tributyroylglycerol. The overlayer gel buffer was 0.1 M Tris-HCl, pH 7.5. Incubation time varied from 2–6 hr at 45 C. Binding of antibodies did not interfere with activity. Lipase activity appeared as cleared zones. In the figures, the cleared zones appear as dark areas, because a dark background was used during photography. Otherwise, the zones cannot be visualized.

Preparation of immobilized lipase A and B. Each of the lipases A (from the conA-Sepharose chromatography) and B (from the Phenyl-Sepharose chromatography) was immobilized on Duolite ES 562 resin (Rohm and Haas, Philadelphia, PA) according to patent application No. 0 140 542. The conditions used for the immobilization were pH 6.0 and 2 hr at 5 C. The intention was to immobilize 14 mg of lipase A preparation and 18 mg of lipase B preparation on 1 gram carrier each. Direct specific activity determinations on these immobilized preparations were not possible. Indirect determinations were done.

Determination of specificities. Positional specificity was determined by thin layer chromatographic (TLC) separation and then visualization of extracts from digestions of trioleoylglycerol. In these, the relative amounts of 1(3)

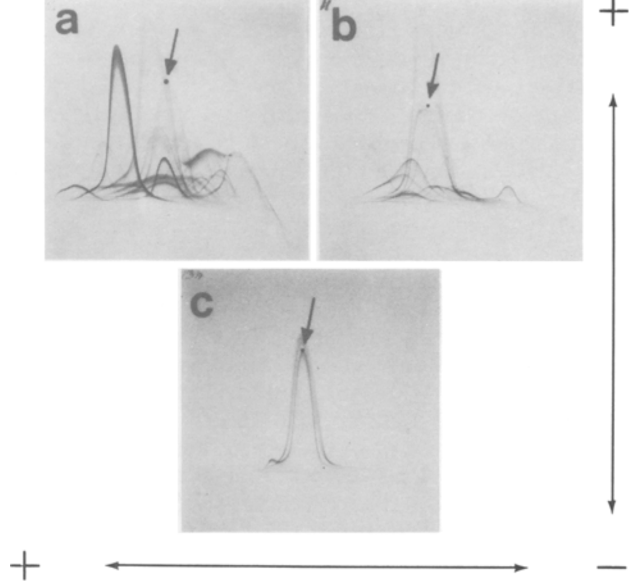

FIG. 3. Crossed immunoelectrophoresis (CIE) of purification steps of lipase B. The antigens applied were as follows: a, 100 μg of crude powder; b, 20 μg of DEAE-Sepharose purified lipase (A/B) preparation; c, 4.4 μg of phenyl-Sepharose purified lipase B preparation. 500 μl of the polyspecific antibodies were applied on each CIE. The CIE were stained with Coomassie Brilliant Blue. The arrow indicates the lipase containing immunoprecipitate, detected as described in Fig. 2.

TABLE 3

Activation Factors[a] of Lipase Preparations Based on LU Values[b]

Purification steps	Lipase preparations A	Lipase preparations B
Crude powder	6.3	6.3
Anion-exchange chromatography	6.3	1.7
Affinity chromatography	5.6	1.1

[a]Activation factor refers to ratio of activities of the enzyme when diluted in glycine buffer, pH 10.5, and in water, respectively, prior to analysis.

[b]LU (lipase unit) is the amount of enzyme that liberates 1 μmol of 4:0/min (see text).

and 2-monooleoylglycerol and 1,2-(2,3) and 1,3-dioleoylglycerol were ascertained by inspection and the real amounts by gas liquid chromatography (GLC) after recovery and conversion to methyl 18:1 with methyl 15:0 as an internal standard (13).

Confirmation was obtained by digestion of the synthetic triacylglycerol (TG), 1,3-dioleoyl-2-palmitoyl glycerol (18:1-16:0-18:1) and 1,3-dipalmitoyl-2-oleoylglycerol (16:0-18:1-16:0). The fatty acids were recovered and identified by GLC (13,14). Fatty acid specificity was determined as described by Wang et al. (15). Equimolar mixtures of the monoacid TG (20 μmol each) of 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 16:0 and 18:1 were hydrolyzed. The residual TG were recovered and separated by temperature-programmed GLC (155 to 350 C) and an 1/8″ × 18″ SS column containing 100/120 mesh Chromosorb coated with 1.0% Dexsil (Supelco, Bellefonte, PA). On-column injection and a cold injector port were required. Nonenzymatic blanks were used to check recoveries of the TG.

For stereospecificity, the specific rotation of the entire extract of a trioleoylglycerol digestion was determined in hexane with a Perkin-Elmer polarimeter sensitive to 1/10000°. In the absence of unequal amounts of optically active 1,2- or 2,3-*sn*-diacylglycerols and with adequate amounts of both, there should be no rotation, hence no stereospecificity (13,16).

Digestions of the substrates were done for the specified times at 40 C. Amounts of enzyme preparations and lengths of incubation were used that would provide 20–60% digestion with times of 1–10 min. For 50 mg of 18:1-18:1-18:1, 16:0-18:1-16:0 and 18:1-16:0-18:1, the following amounts of enzymes were used: soluble A, 88 LU or 58 NLU; soluble B, 94 LU or 59 NLU; immobilized A, 10 mg equivalent to 0.14 mg of Soluble A; immobilized B, 10 mg equivalent to 0.18 mg of soluble B. The buffer was 0.25 M Tris (pH 8.0) containing 1.0% gum arabic. The substrates were emulsified, after being melted when necessary, with a Branson sonicator. The substrates 18:1-16:0-18:1 and 16:0-18:1-16:0 were synthesized as described by Jensen and Pitas (17). Analyses with pancreatic lipase have shown that TG synthesized by these methods are 98–99% positionally correct. The monoacid TG of 4:0, 6:0, 8:0, 10:0, 12:0, 14:0, 16:0 and 18:1 were purchased from Sigma Chemical Co. (St. Louis, MO), and purity was determined by TLC (17).

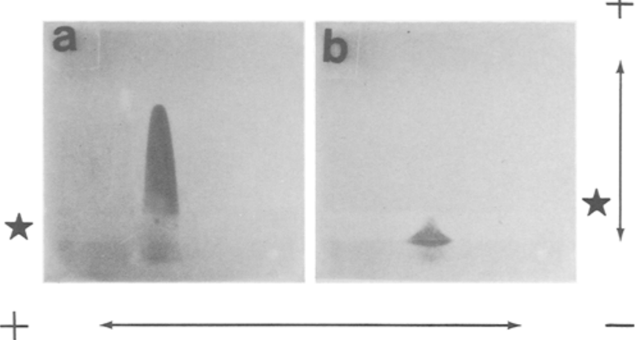

FIG. 4. Crossed immunoaffinity electrophoresis (CIAE), with tributyroylglycerol overlayers. Lipase peaks show as clearing zones. The intermediate gel (★) was without conA-Sepharose in a and contained conA-Sepharose in b. Applied antigen was 100 μg of crude powder on each CIAE. Applied antibodies as in Figs. 2 and 3. A typical CIAE of lipase A (a similar pattern is seen for the conA-Sepharose purified lipase preparation).

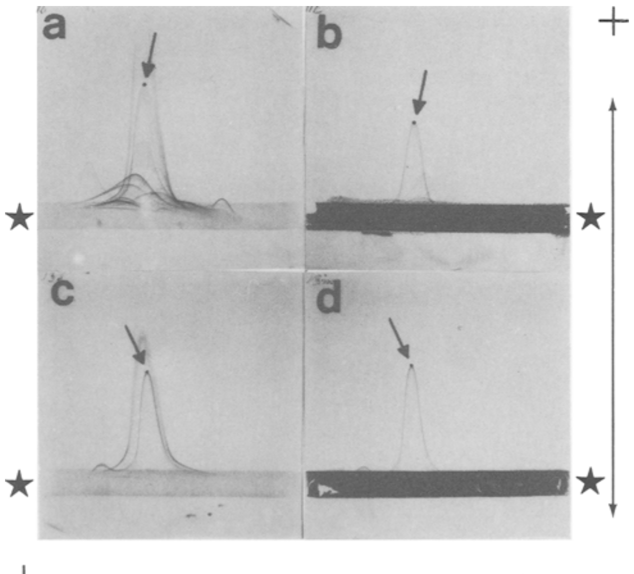

FIG. 5. Crossed immunoaffinity electrophoresis (CIAE), stained with Coomassie Brilliant Blue. The arrow indicates the lipase containing immunoprecipitate, detected as described in Fig. 2. The antigens applied were as follows: a and b, respectively, 20 μg of DEAE-Sepharose purified lipase A/B preparation; c and d, respectively, 4.4 μg of phenyl-Sepharose purified lipase B preparation. Applied antibodies as Figs. 2 and 3. The intermediate gels (★) were without conA-Sepharose in a and c and contained conA-Sepharose in b and d. This figure together with Fig. 4 shows decreasing affinity to conA-Sepharose of lipase, when it is converted from the A form to the B form.

RESULTS AND DISCUSSION

Conversion of the lipase from the A form to the B form was done by keeping the lipase fractions obtained from purification steps 1 and 2 at low pH during recovery and by treating the enzyme at low pH at an elevated temperature prior to step 2 as described previously. Lipase activity bands and peaks in IEF and CIE/CIAE, respectively, were identified by performing duplicates of the electrophoretic runs. These duplicates were used for the substrate overlayers and compared with the Coomassie-

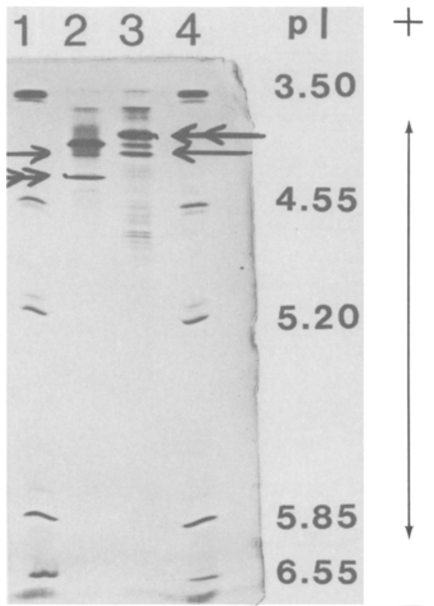

FIG. 6. Isoelectric focusing (IEF) of lipase A and B, stained with Coomassie Brilliant Blue. Lanes 1 and 4, respectively, about 65 μg of the total reference proteins. Lane 2, about 40 μg of the phenyl-Sepharose purified lipase B preparation; lane 3 about 40 μg of the conA-Sepharose purified lipase A preparation. Double arrow: main lipase activity band; single arrow: weak lipase activity band, as detected by means of tributyroylglycerol overlayer on a duplicate IEF. The isoelectric points (pI) of these bands were estimated on the basis of the reference proteins.

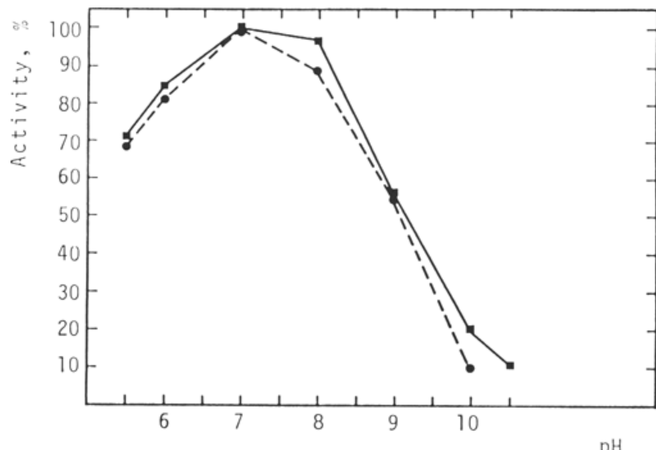

FIG. 7. Effect of pH on activity on tributyroylglycerol, 30 C, pH-stat method (7) using the appropriate analysis pH values. (■) Lipase A, conA-Sepharose purified preparation. (●) Lipase B, phenyl-Sepharose purified preparation.

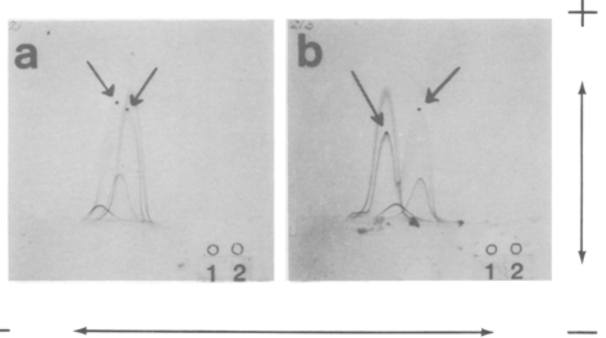

FIG. 8. Tandem-crossed immunoelectrophoresis, stained with Coomassie Brilliant Blue. Arrows indicate lipases, detected as described in Fig. 2. Applied antibodies as in Fig. 2. (a) Well 1, 5 μg of conA-Sepharose purified lipase A preparation. (b) Well 1 and well 2, reverse order of (a).

stained plates. The purifications were followed by activity measurements on both 4:0-4:0-4:0 and olive oil as well as by the electrophoretic methods SDS-PAGE and CIE. Tables 1 and 2 summarize the purification steps and yields of lipases A and B, respectively. Figures 1, 2 and 3 show the successive purifications of lipase from the multiprotein mixture in the crude powder with removal of many nonlipase proteins in the starting material. In Figure 3c, the antigenically different protein is not a lipase. Since the conversion of A to B was incomplete, the intermediate A/B appears as a broad peak in Figure 3b.

The differences in lipolytic and molecular behavior of the lipases were investigated by determining activities on water dilutions of the enzyme preparations as well as by CIAE. In Table 3 we present data on differences between lipases A and B in activity ratios when they are diluted in glycine buffer, pH 10.5, and in water, respectively, prior to analysis. It is obvious that lipase B needs no "activation" at high pH, whereas lipase A does. In Figures 4 and 5 we show the relative affinities of lipases A and B toward conA-Sepharose. A typical CIAE of lipase A, which had a high degree of affinity to conA-Sepharose, indicating that the lipase is a glycoprotein, is shown in Figure 4. The marked decreasing affinity to conA-Sepharose, when the lipase is converted from A form to the B form (A/B) due to partial deglycosylation, is given in Figure 5.

In Figure 5 (IEF), we depict the different isoelectric points (pI) of lipases A and B. The pI of the main lipase band in the lipase A preparation is 3.9, whereas it is 4.3 in the lipase B preparation. The more distinct band in B has a pI of 4.0, but does not have lipase activity. These results, combined with the findings of the slightly different net charges at pH 8 (cf. Fig. 8), indicate that the titration curves of the two lipases cross each other somewhere between pH 8.0 and 4.3.

Effect of pH on activities of lipase A and B on tributyroylglycerol was also investigated using the appropriate analysis pH-values in Novo method AF 95.1/3 GB. Figure 7 shows similar pH-activity profiles of the two lipases, both having optima at pH 7.0. Only a minor difference is seen at pH 8.0, where lipase B has slightly lower activity than lipase A.

Figure 8 (tandem-CIE) clearly shows a high degree of antigenic identity between lipases A and B, as defined in ref. 12. Their immunoprecipitates fuse into a double peak, practically without any spurs. A difference in the morphology of the immunoprecipitates is seen, though. The reverse orders of the well positions of lipase A and B in the first dimension gel also visualize the greater anodic mobility of B.

The results indicate that the B-form of the lipase is derived by partial deglycosylation of the A-form and that this influences the activity mode on emulsions. Lipase B is the "activated" form, but by the recovery yields in Ta-

TABLE 4

Composition of Fatty Acids Produced by Digestion of 18:1-16:0-18:1[a] and 16:0-18:1-16:0 with Several Preparations of *Mucor miehei* Lipase[b]

Substrate and composition of fatty acids (mol%)	Lipase preparation			
	Soluble A	Immobilized A	Soluble B	Immobilized B
18:1-16:0-18:1				
16:0	17.7	17.9	20.3	17.2
18:1	82.3	83.1	79.7	82.8
16:0-18:1-16:0				
16:0	90.9	93.2	91.5	94.7
18:1	9.1	6.8	8.5	5.3

[a]Average of 5 determinations.
[b]18:1-16:0-18:1 is 1,3-dioleoyl-2-palmitoyl-glycerol.

TABLE 5

Digestion (%) of Equimolar Mixtures of Monoacid Triacylglycerols by Lipase Preparations from *Mucor miehei*

Length of digestion (min)	Monoacid triacylglycerol, % digested							
	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:1
Soluble A								
1	35.6	38.2	43.4	40.1	47.4	47.1	45.3	50.0
2	42.4	46.5	52.8	48.9	54.9	53.8	50.7	53.7
3	54.2	61.1	65.4	61.0	65.7	64.7	62.7	62.2
5	61.0	66.7	73.6	70.3	76.6	75.6	76.6	74.5
Soluble B								
1	27.6	33.4	40.7	40.7	44.3	45.8	39.4	38.0
2	32.1	49.2	51.4	57.7	63.1	63.4	62.4	56.7
3	74.6	73.1	78.8	78.8	81.8	51.5	80.7	78.9
5	87.5	86.5	81.4	89.3	92.8	92.1	91.7	90.9
Immobilized A								
1	31.8	30.6	35.6	39.3	42.8	57.4	52.3	43.8
2	43.9	42.0	49.5	54.8	54.6	61.5	63.2	55.7
3	—	43.3	52.1	56.0	57.7	64.3	65.4	56.6
5	65.2	65.6	72.9	74.6	76.4	79.7	82.7	75.8
Immobilized B								
1	25.4	17.5	18.3	14.2	21.2	18.7	21.2	10.4
2	36.6	28.5	26.9	20.9	22.9	37.5	36.0	29.8
3	39.7	39.7	47.0	44.2	52.9	57.5	55.9	57.8
5	42.9	45.3	51.2	47.2	57.7	61.9	59.2	59.9

bles 1 and 2 it seems to be less stable than lipase A. We postulate that this partial deglycosylation at low pH of the original lipase is due to enzymatic reactions.

All preparations were highly specific for the primary positions of 18:1-18:1-18:1 as indicated by the absence of 1(3)-monoacylglycerols (MG) and 1,3-diacylglycerols (DG) in boric acid-TLC plates of extracts from short digestions of 10 min. The profiles of digestion products were similar for all preparations; rapid appearance of free fatty acids and 1,2(2,3)-DG accompanied by disappearance of TG at 4 and 8 min. Not much MG was produced at 4 min, but the amounts increased dramatically at 8 min, reflecting the accumulation of 2-MG. These will convert to 1-MG by acyl migration and will eventually be hydrolyzed, resulting in glycerol, which we did not determine. By 8 min, 30–50% of the TG was gone and by 12 min the amounts were less than 10%.

Confirmation of specificity for the primary positions of TG was obtained by analyses of the fatty acids liberated by the lipolyses of 18:1-16:0-18:1 and 16:0-18:1-16:0 (Table 4). In both cases, all preparations released much greater amounts (79.7–94.7 mol%) of the acids in the primary

positions than 67 mol%, the quantity that would be produced by a nonspecific lipase (13). Interestingly, there was a slight preference for 16:0 as compared to 18:1 in the primary positions.

We analyzed the whole extracts of digestions of 18:1-18:1-18:1 with a sensitive polarimeter but found no deviations from zero specific rotation (13). We used this technique in our study on human lingual lipase (16), finding a rotation denoting the presence of *sn*-1,2-DG. It is possible that enantiomeric MG as well as DG could be responsible for rotation, but since we observed no rotation, further investigation was not needed.

In contrast to Wang et al., who found specificity by a breast milk lipoprotein lipase (15) and a bile salt–stimulated lipase (18) for the shorter fatty acids in mixtures of monoacid TG ranging from 4:0 to 18:1, we observed the opposite. The percent digestions of the 4:0, 6:0, 8:0 and 10:0 TG were almost always lower than those of 12:0, 14:0, 16:0 and 18:1 with all preparations except Sol B. These data are presented in Table 5. In contrast to soluble A, immobilized A released more 14:0 and 16:0 than the other acids, including 18:1. This also occurred with the B preparations, but to a lesser extent. It is possible that the enzymes showed some preference for 14:0 and 16:0. This is supported by the results from the digestions of 16:0-18:1-16:0 and 18:1-16:0-18:1, previously mentioned and also in Table 4.

In summary, there are distinct differences in some characteristics including specificity for fatty acids among the several preparations. Neither deglycosylation of *M. miehei* lipase A to produce B nor immobilization of either preparation altered other specificities studied.

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Effects of Triton WR 1339 and Orotic Acid on Lipid Metabolism in Rats

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In order to investigate the effect of hepatic cholesterol flux on biliary bile acids, Triton WR 1339 and orotic acid were administered to rats, and the biliary cholesterol, phospholipids and bile acids were analyzed together with serum lipoproteins and hepatic lipids. Triton, which raised serum very low density lipoprotein and lipid levels and decreased serum high density lipoprotein liver lipid levels, increase the biliary cholic acid group/chenodeoxycholic acid group ratio (CA/CDCA) in the bile without affecting the total amount of bile acids and the other biliary lipids. Orotic acid, which decreased serum lipid and lipoprotein concentrations and increased liver lipid levels, increased the biliary excretion of cholesterol and phospholipids, but produced no significant change in the total amount of bile acids and in the CA/CDCA ratio in bile.
Lipids 22, 566–571 (1987).

The liver plays a central role in the regulation of the cholesterol metabolism. A considerable portion of cholesterol in the body pool is synthesized in the liver and released into the blood serum in the form of very low density (VLDL) and high density (HDL) lipoprotein. The liver, on the other hand, takes up cholesterol from plasma, metabolizes it to bile acids and secretes these into bile. This cholesterol flux from the liver and plasma is considered to influence cholesterol degradation and synthesis. Increased efflux of cholesterol from the liver enhances hepatic cholesterol synthesis, while increased influx inhibits it (1,2).

Precursor cholesterol for biliary cholesterol and bile acids is supplied from at least three sources: newly synthesized cholesterol in the liver, stored cholesterol in the liver and cholesterol derived from plasma lipoproteins. It has been suggested that newly synthesized hepatic cholesterol is a preferred source for bile acids (3–5). However, 10–20% of biliary cholesterol is estimated to arise from the newly synthesized pool of cholesterol (6,7), although larger amounts of cholesterol and bile acids have been derived from the newly synthesized cholesterol after prolonged bile drainage (6–8). Therefore, some manipulation of cholesterol and bile acid metabolism, such as cholesterol feeding (9,10), administration of bile acid sequestering resins (11–14) or bile drainage (6,8), should change the contribution of newly synthesized cholesterol to biliary cholesterol and bile acids.

Triton WR 1339 is known to reduce the influx of plasma cholesterol into the liver (15–19), and orotic acid is known to inhibit the efflux of hepatic cholesterol (20,21). These changes in the cholesterol flux between liver and plasma affect bile acid metabolism. Therefore, in the present experiments, we administered Triton WR 1339 and orotic acid to rats and examined the changes in biliary cholesterol, phospholipids and bile acids as well as in serum lipoprotein and liver lipid levels.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing about 250 g were housed in an air-conditioned room under artificial light from 7 a.m. to 7 p.m. and were given a balanced regular stock diet (Type MF, Oriental Kobo Co., Tokyo). Triton WR 1339 at 40 mg/100 g body weight was dissolved in 0.5 ml saline and injected intraperitoneally (ip) to interrupt cholesterol influx into the liver. Control rats received the same volume of saline ip. After 20-hr fasting, the rats were used for the experiments.

In another set of experiments, 15 g of powdered regular diet supplemented with 2% orotic acid was given to rats daily for 7 days to inhibit the cholesterol efflux from the

TABLE 1

Serum Lipid Concentrations of Rats Treated with Triton WR 1339 and with Orotic Acid

Serum lipids	Control (13)[a]	Triton (15)	Control (10)	Orotic acid (5)
Total cholesterol	68.1 ± 2.42[b]	423.7 ± 27.76[c]	56.4 ± 2.32	21.9 ± 4.02[c]
Free cholesterol	10.2 ± 0.86	320.8 ± 15.04[c]	11.2 ± 0.64	5.3 ± 0.76[c]
Esterified cholesterol	57.8 ± 2.86	102.9 ± 9.52[c]	45.2 ± 2.43	16.6 ± 3.32[c]
HDL cholesterol	47.5 ± 3.88	4.3 ± 0.25[c,*]	36.8 ± 1.69	18.2 ± 2.51[c]
Triglycerides	196.2 ± 25.21	2857 ± 204.7[c]	183.9 ± 13.61	33.6 ± 5.84[c]
Phospholipids	120.8 ± 6.23	627.1 ± 51.48[c]	89.0 ± 4.93	31.0 ± 5.89[c]

[a]Number of rats.
[b]Mean ± S.E.M. (mg/100 ml).
[c]Significant difference: $p < 0.001$.
*n = 10, because HDL cholesterol could not be separated in five cases.

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Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; CA, cholic acid; CDCA, chenodeoxycholic acid; LCAT, cholesteryl acyltransferase.

TABLE 2

Compositions of Serum Lipoproteins from Rats Treated with Triton WR 1339 and with Orotic Acid

Lipoproteins	Control (13)[a]	Triton (15)	Control (10)	Orotic acid (5)
VLDL	224.2 ± 35.68[b]	3760.5 ± 299.02[c]	203.1 ± 18.34	21.6 ± 3.36[c]
Protein	18.6 ± 1.15	120.0 ± 3.69[c]	20.9 ± 1.55	1.6 ± 0.50[c]
Free cholesterol	7.0 ± 1.05	193.1 ± 13.69[c]	4.6 ± 0.54	0.2 ± 0.12[c]
Esterified cholesterol	6.3 ± 0.57	79.7 ± 17.01[c]	3.1 ± 0.35	0.2 ± 0.08[c]
Triglycerides	157.0 ± 28.29	2819.2 ± 25.00[c]	147.2 ± 13.60	17.1 ± 2.44[c]
Phospholipids	34.3 ± 4.37	548.5 ± 39.79[c]	27.4 ± 2.72	2.6 ± 2.72[c]
LDL	56.5 ± 3.70	108.3 ± 8.04[c]	42.7 ± 0.93	9.6 ± 1.82[c]
Protein	15.6 ± 0.57	26.9 ± 1.28[c]	12.9 ± 1.74	2.5 ± 0.65[c]
Free cholesterol	2.2 ± 0.24	11.0 ± 0.91[c]	1.3 ± 0.12	0.3 ± 0.13[c]
Esterified cholesterol	13.6 ± 0.29	19.7 ± 2.11[c]	7.3 ± 0.30	1.1 ± 0.39[c]
Triglycerides	9.9 ± 1.76	20.0 ± 1.87[c]	9.5 ± 0.32	3.9 ± 0.36[c]
Phospholipids	15.2 ± 0.92	30.7 ± 1.87[c]	11.7 ± 0.47	1.8 ± 0.34[c]
HDL	198.8 ± 5.29	41.1 ± 8.53[c]	179.7 ± 6.49	88.9 ± 9.63[c]
Protein	97.9 ± 3.94	20.9 ± 4.17[c]	95.6 ± 3.06	51.6 ± 4.31[c]
Free cholesterol	3.5 ± 0.07	1.6 ± 0.35[d]	2.7 ± 0.19	2.3 ± 0.39
Esterified cholesterol	39.8 ± 1.38	4.9 ± 1.47[c]	33.2 ± 1.88	17.7 ± 2.62[c]
Triglycerides	8.8 ± 0.67	6.7 ± 0.44[c]	8.1 ± 0.53	3.5 ± 0.15[c]
Phospholipids	49.0 ± 2.13	6.9 ± 2.46[c]	40.2 ± 3.31	13.8 ± 2.62[c]

[a]Number of rats.
[b]Mean ± S.E.M. (mg/100 ml).
[c]Significant difference: $p < 0.001$.
[d]Significant difference: $p < 0.01$.

liver. Control rats were fed the same amount of regular diet without orotic acid. On the last day, 15 g of the diet containing 2% orotic acid or the control diet was given to the rats 12 hr prior to the experiment.

Bile was collected by biliary drainage for 30 min under sodium pentobarbital anesthesia (50 mg/kg, ip) (9), and then blood was drawn from the abdominal aorta by syringe. The liver was removed immediately after perfusion with 10 ml of cold saline through the portal vein.

Serum total and free cholesterol (22), triglycerides (23) and phospholipids (24) were determined by enzymatic colorimetric tests, and HDL cholesterol was estimated by an enzymatic method after removal and VLDL and low density lipoprotein (LDL) by the addition of phosphotungstic acid and $MgCl_2$ (25).

Serum lipoproteins were separated by ultracentrifugation as described by Hatch and Lees (26). VLDL was obtained by flotation of half the volume of the serum sample. For this purpose, NaCl solution with a density of 1.006 g/100 ml was layered on top of the serum and centrifuged at 100,000 × g for 16 hr in a Hitachi 65P ultracentrifuge. Half the volume of the NaCl solution on top of the serum was removed as the VLDL fraction. LDL was separated by centrifugation at 100,000 × g for 20 hr after adjusting the density to 1.064 by addition of KBr solution with a density of 1.182. HDL was obtained after centrifugation at 100,000 × g for 40 hr from the serum adjusted to a density of 1.21 by addition of KBr solution with a density of 1.478. In the case of the Triton-treated rats, VLDL separations were repeated three times to obtain the VLDL fraction completely. Lipid concentrations in the lipoprotein fractions were determined by enzymatic colorimetric assays (22–24), and apoprotein concentrations were measured by the Lowry method (27).

Cholesterol and bile acids in the bile were analyzed by gas liquid chromatography (28), and phospholipids were determined by the method of Haeflmyar and Fried (29) after extraction with hot ethanol. Lithogenic indices of the biles were calculated by the methods of Admirand and Small (30) and Holzbach et al. (31). Liver lipids were extracted by the Folch procedure (32); total lipids, total cholesterol, triglycerides and phospholipids were determined as reported previously (33). Statistical analyses were carried out using Student's t-test.

RESULTS

Table 1 shows the serum lipid concentrations of the rats treated with Triton and with orotic acid. The serum triglyceride concentration in the Triton-treated rats increased to about 15 times that of the control rats, and the total cholesterol and phospholipids increased 6.2 and 5.2 times, respectively. By contrast, the HDL cholesterol decreased to about one-tenth of that of the control, suggesting that the serum cholesterol was contained exclusively in the VLDL and LDL fractions after the treatment with Triton. As the serum-free cholesterol concentration was elevated to 31.5-fold that of the control, the percentage of the esterified cholesterol decreased to 24.3%, while the control showed a value of 84.9%.

On the other hand, orotic acid markedly decreased the serum lipid levels. The total cholesterol, triglycerides and phospholipids showed values of 38.8, 18.3 and 34.8% of those of the control rats, respectively. The HDL choles-

TABLE 3

Percentages of Lipoprotein Compositions from Rats Treated with Triton WR 1339 and with Orotic Acid

Lipoproteins	Control (13)[a]	Triton (15)	Control (10)	Orotic acid (5)
VLDL				
Protein	8.3±0.71[b]	3.2±0.12[c]	10.3±0.50	7.4±1.12[d]
Free cholesterol	3.1±0.67	5.1±0.18[c]	2.3±0.13	0.9±0.11[c]
Esterified cholesterol	2.8±0.23	2.1±0.07	1.5±0.14	0.9±0.23[e]
Triglycerides	70.0±1.12	75.0±0.81[d]	72.5±0.58	79.2±1.41[c]
Phospholipids	15.3±0.30	14.6±0.20	13.5±0.38	12.0±0.37[e]
LDL				
Protein	27.6±0.69	24.8±0.58[e]	30.2±1.61	26.0±1.97
Free cholesterol	3.9±0.17	10.2±0.25[c]	3.0±0.23	3.1±0.64
Esterified cholesterol	24.1±0.61	18.2±0.58[c]	17.1±0.40	11.5±1.72[d]
Triglycerides	17.5±0.99	18.5±1.07	22.2±0.87	40.6±4.39[c]
Phospholipids	26.9±0.26	28.3±0.58	27.4±0.98	18.8±1.47[e]
HDL				
Protein	49.2±1.03	50.9±1.51	53.2±1.28	58.0±1.56[e]
Free cholesterol	1.8±0.44	3.9±0.32[d]	1.5±0.07	2.6±0.26[d]
Esterified cholesterol	20.0±0.54	11.9±1.29[c]	18.5±1.28	19.9±0.97
Triglycerides	4.4±0.43	16.3±2.16[c]	4.5±0.42	3.9±0.39
Phospholipids	24.6±0.74	16.8±2.03[c]	22.4±1.37	15.5±1.59[e]

[a]Number of rats.
[b]Mean ± S.E.M. (mg/100 ml).
[c]Significant difference: $p < 0.001$.
[d]Significant difference: $p < 0.01$.
[e]Significant difference: $p < 0.05$.

TABLE 4

Hepatic Lipid Concentrations from Rats Treated with Triton WR 1339 and with Orotic Acid

	Control (8)[a]	Triton (12)	Control (10)	Orotic acid (5)
Liver weight	5.06±0.11[b]	4.46±0.13[e]	4.84±0.11	5.87±0.17[e]
Triglycerides	4.90±0.59[c]	3.48±0.33[e]	6.53±1.19	24.26±0.64[d]
Cholesterol	1.64±0.12[c]	1.49±0.08	1.96±0.10	2.70±0.19[e]
Phospholipids	22.46±1.78[c]	22.17±1.44	26.86±1.19	30.42±1.65
Total lipids	34.23±1.81[c]	28.10±1.47[e]	38.02±0.91	63.50±4.26[d]

[a]Number of rats.
[b]Mean ± S.E.M. (g/100 g body weight).
[c]Mean ± S.E.M. (mg/g wet tissue).
[d]Significant difference: $p < 0.001$.
[e]Significant difference: $p < 0.01$.

terol was also decreased to 49.4% of the control, suggesting that the cholesterol in VLDL and LDL decreased to about one-fifth of that of the control. The percentage of the esterified cholesterol vs total cholesterol was not affected by orotic acid administration.

The effect of Triton on apoproteins and lipids in serum lipoproteins is shown in Tables 2 and 3. The VLDL concentration in the treated rats increase 16.8-fold over the control, and the percentages of free cholesterol and triglycerides in VLDL increased while that of apoproteins decreased. The LDL concentration and free cholesterol increased, but apoproteins and esterified cholesterol decreased. The HDL concentration, on the contrary, markedly decreased to about one-fifth of that of the control. Free cholesterol and triglycerides in HDL increased, those of esterified cholesterol and phospholipids decreased, and that of apoproteins remained unchanged.

The VLDL concentration in the rats fed orotic acid decreased to one-tenth of that of the control, as shown in Table 2, where the composition ratios of the surface components such as apoproteins, free cholesterol and even phospholipids decreased but that of triglycerides in-

creased (Table 3). The LDL concentration also decreased with the reduction in the composition ratios of esterified cholesterol and phospholipids and with an increase in the triglycerides. The HDL concentration decreased to about half of the control; the percentage of free cholesterol increased but that of phospholipids decreased.

The liver weight of the Triton-treated rats decreased to 9.5 ± 0.27 g from 11.1 ± 0.29 g in the control ($p < 0.01$), but that of the orotic acid–treated rats increased to 16.3 ± 0.78 g from 14.1 ± 0.36 g in the control ($p < 0.01$). Triton decreased the total lipid level in the liver, but orotic acid markedly increased it (Table 4). Although only the triglyceride concentration decreased in the liver of Triton-treated rats, both the triglyceride and total cholesterol concentrations in the orotic acid–treated rats increased markedly.

Table 5 shows the changes in bile flow and levels of cholesterol, phospholipid and bile acid in the Triton- and orotic acid– treated rats. Triton induced no change in the bile flow and biliary cholesterol, phospholipid and bile acid levels, but orotic acid increased the bile flow and biliary secretion of cholesterol and phospholipids. The bile acid secretion in the orotic acid–treated rats showed a tendency to increase, but the change was statistically insignificant.

Lithogenic indices calculated by the methods of Admirand and Small (30) and Holzbach et al. (31) were not affected in the Triton treatment. In the orotic acid–treated rats, maximal cholesterol solubilities estimated by both methods tended to increase. Lithogenic indices therefore were not altered significantly by orotic acid administration despite the tendency to increase.

The biliary bile acid composition in these rats is given in Table 6. In the Triton-treated rats, 3α,12β-dihydroxy-7-oxo-5β-cholanoic acid increased and β-muricholic acid decreased, resulting in an increase of the cholic acid (CA) group and a decrease of the chenodeoxycholic acid (CDCA) group. The increase of cholic acid and deoxycholic acid and the decrease of chenodeoxycholic acid were statistically insignificant, but the ratio of the CA/CDCA group increased in the Triton-treated rats (2.19 vs. 1.54, $p < 0.05$). On the other hand, orotic acid caused no significant change in the biliary bile acid composition.

DISCUSSION

Both Triton WR 1339 and orotic acid affect the distribution of lipids between plasma and liver. Triton reduced the hepatic uptake of lipids from plasma lipoproteins by inhibiting the interaction between VLDL and lipoprotein

TABLE 5

Effects of Triton WR 1339 and Orotic Acid on Biliary Lipids

	Control (9)[a]	Triton (6)	Control (7)	Orotic acid (5)
Bile flow				
ml/hr	1.10 ± 0.06[b]	1.10 ± 0.11	1.53 ± 0.09	1.92 ± 0.15[c]
Cholesterol				
%g/ml	232 ± 21.5	232 ± 29.9	123 ± 13.7	156 ± 13.5
%g/hr	251 ± 30.5	263 ± 30.8	192 ± 27.1	301 ± 42.6[c]
Phospholipids				
μg/ml	2.58 ± 0.17	2.69 ± 0.34	2.56 ± 0.24	2.85 ± 0.22
μg/hr	2.92 ± 0.22	3.19 ± 0.29	3.94 ± 0.45	5.39 ± 0.51[c]
Bile acids				
μg/ml	8.92 ± 0.85	9.01 ± 1.23	8.49 ± 1.01	7.42 ± 0.51
μg/hr	8.99 ± 0.33	9.02 ± 1.29	12.9 ± 1.64	14.00 ± 0.29
Molar percent				
Cholesterol	2.3 ± 0.23	2.3 ± 0.26	1.3 ± 0.20	1.9 ± 0.21
Phospholipids	13.9 ± 1.34	14.6 ± 2.02	13.9 ± 1.60	16.9 ± 1.01
Bile acids	83.8 ± 1.54	83.0 ± 2.23	84.8 ± 1.80	81.3 ± 1.14
PL/(PL + BA)	0.142 ± 0.013	0.149 ± 0.018	0.147 ± 0.015	0.172 ± 0.010
Admirand and Small's method				
MCS	8.78 ± 0.279	8.84 ± 0.402	8.43 ± 0.406	9.30 ± 0.160
LI	0.261 ± 0.023	0.257 ± 0.024	0.171 ± 0.016	0.201 ± 0.021
Holbach et al. method				
MCS	4.79 ± 0.260	4.98 ± 0.385	4.87 ± 0.294	5.37 ± 0.226
LI	0.477 ± 0.032	0.462 ± 0.039	0.276 ± 0.023	0.359 ± 0.033

MSC, Maximal cholesterol solubility; LI, lithogenic index.

[a]Number of rats.

[b]Mean ± S.E.M.

[c]Significant difference: $p < 0.05$.

TABLE 6

Effects of Triton WR 1339 and Orotic Acid on Composition of Biliary Bile Acids

	Control (9)[a]	Trinton (6)	Control (7)	Orotic acid (5)
CA group	52.1±1.94[b]	60.7±2.17[c]	51.2±2.39	54.7±2.41
Deoxycholic	2.9±0.34	4.3±0.66	2.7±0.38	1.5±0.30
Cholic	46.1±2.47	48.3±2.62	46.2±2.32	50.6±2.26
3α,12β,7=0	3.0±0.88	8.0±2.05[c]	2.7±0.79	2.6±0.64
CDCA group	33.81±1.15	27.7±1.67[c]	35.4±1.84	31.6±1.32
Lithocholic	0.4±0.03	0.4±0.09	0.4±0.05	0.5±0.07
Chenodeoxycholic	6.0±0.65	5.1±1.25	5.4±0.71	4.7± 1.54
Hyodeoxycholic	1.4±0.29	1.6±0.37	2.6±0.14	1.8±0.25
Ursodeoxycholic	2.8±0.34	2.2±0.23	2.2±0.41	1.5±0.09
α-Muricholic	2.9±0.29	2.8±0.75	2.8±0.41	3.6±0.46
β-Muricholic	13.6±1.01	9.2±1.01[c]	16.2±1.83	12.3±0.61
ω-Muricholic	1.1±0.12	1.3±0.15	1.8±0.13	1.5±0.25
3α,6=0	5.6±0.74	5.1±0.42	4.0±0.64	5.6±0.44
Others	14.2±1.16	11.8±1.58	11.5±1.39	12.5±0.94
CA/CDCA ratio	1.54±0.12	2.19±0.23[c]	1.48±0.15	1.73±0.14

[a]Number of rats.
[b]Mean ± S.E.M. (%).
[c]Significant difference: $p < 0.05$.

lipase (15–17) or between HDL and lecithin: cholesterol acyltransferase (LCAT) (18,19).

Elevation of the percentages of triglycerides and cholesterol in the VLDL fraction indicate an increase of large-sized VLDL, from which lipid moieties are not eliminated by lipoprotein lipase. The marked increase of VLDL causes some VLDL contamination of other lipoprotein fractions. Despite repeated separation of VLDL, a relatively triglyceride-rich HDL is obtained, because some triglyceride appears to be transferred from VLDL to HDL (34–36). It also will generate LDL particles in which esterified cholesterol in the core portion is substituted for triglyceride.

The decreased percentage of esterified cholesterol in serum indicates an impairment of LCAT activity, but the decrease of HDL concentration brought about by the reduction of HDL formation from VLDL (37–39) is also responsible for the reduction of the LCAT effect, because cholesterol is esterified on HDL surfaces (35,40,41). Since cholesterol is transferred from HDL to VLDL and LDL (34–36), the reduction of the esterified cholesterol content in HDL should lead to a decrease in the percentage of esterified cholesterol in VLDL and LDL.

It is not clear why the content of triglyceride in VLDL and LDL was elevated but those of protein and lipid were decreased by orotic acid administration. Orotic acid is known to specifically inhibit the release of VLDL from liver, but does not affect the intestinal relaease of chylomicrons and VLDL (42,43). Since chylomicron production in the intestine may be negligible after 12-hr fasting of rats and LDL may not be transformed from chylomicron, intestinal lipoproteins may not have participated in such compositional changes in VLDL and LDL in the present experiment. Therefore, the metabolism of triglyceride-rich lipoproteins from the liver should be affected indirectly by orotic acid.

Since Triton treatment inhibits cholesterol uptake by the liver from plasma without interfering with VLDL secretion from the liver (44,45), the hepatic cholesterol content is reduced, which in turn stimulates cholesterol synthesis in the liver. Actually, hepatic lipid synthesis was shown to be enhanced by Triton administration (15,46,47), although some reports have shown that the lipid synthesis was not affected (16,48,49). More recently, Fears and Umpleby (50) reported that hepatic cholesterol synthesis was stimulated, but the triglyceride synthesis remained unchanged after Triton treatment. In the present experiment, liver triglyceride decreased after the treatment but cholesterol did not, suggesting that the compensatory synthesis of cholesterol is more sensitive than that of triglyceride. Since phospholipids, which are an important membrane constituent of cells, are contained in VLDL far less than triglycerides, the effect of Triton treatment on phospholipids will be less significant than that on triglycerides. Consequently, newly synthesized or stored cholesterol in the liver will become the preferred precursor for bile acid synthesis. In addition, it has been reported that newly synthesized cholesterol in the liver is preferably metabolized to CA rather than to CDCA (3,51,52). Glucose feeding after fasting (53), oral administration of bile acid sequesting resin (11,14) or phytosterol (54), and bile drainage (6,8) stimulate bile acid, especially CA, synthesis. In the present experiment, the CA/CDCA ratio of the biliary bile acids was increased by Triton administration, suggesting that the CA synthetic pathway is stimulated by the compensatory increase of cholesterol synthesis.

On the other hand, orotic acid is considered to inhibit the glycosylation of apolipoproteins in the liver (20) and to decrease the release of VLDL into the circulation, inducing fatty liver and hypolipidemia in rats (20,21). VLDL and HDL are formed in both the liver and intestine, but VLDL secretion from the liver is five to eight times faster than that from the intestine (55), while a considerably larger amount of HDL is secreted from the intestine (56). Therefore, the decrease in serum VLDL should be

more marked than that of HDL in the orotic acid–treated rats. In addition, HDL formation from VLDL should be reduced when the serum VLDL level is low. This may cause a decrease of serum HDL after orotic acid treatment.

In the present experiment, the biliary bile acid secretion and CA/CDCA ratio were not influenced by orotic acid, but the secretions of cholesterol and phospholipids increased. Cholesterol feeding in excess to rats also causes accumulation of cholesterol and triglycerides in the liver and increases biliary secretions of bile acids and cholesterol (9,10). Thus, cholesterol accumulation in the liver probably enhances the excretion of biliary cholesterol. As for the bile acid composition, cholesterol feeding stimulates the CDCA synthetic pathway and leads to decrease in the CA/CDCA ratio of the bile (9,10). In the present experiment, the CA/CDCA ratio as well as the total amounts of excreted bile acids were not affected significantly in orotic acid–treated rats. Although accumulation of cholesterol in the liver is caused by either an increase of cholesterol influx or a decrease of cholesterol outflux, and the bile acid metabolism is influenced by the increase of liver cholesterol, the change of bile acid metabolism produced by the increase of cholesterol influx might be different from that induced by the decrease of cholesterol efflux. However, Tokmakjian and Haines (57) recently reported that orotic acid stimulated hepatic cholesterol synthesis. Although we expected that the CA/CDCA ratio would decrease in the orotic acid–treated rats, our results did not show this. If orotic acid does increase the cholesterol synthesis in the liver, as they have shown, this might have cancelled the decrease in the CA/CDCA ratio.

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Alteration of Hepatic Phospholipids in Rats and Mice by Feeding Di-(2-Ethylhexyl)Adipate and Di-(2-Ethylhexyl)Phthalate

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Effects of di-(2-ethylhexyl)adipate (DOA) and di-(2-ethylhexyl)phthalate (DEHP), plasticizers for polyvinylchloride products, on concentrations and compositions of hepatic phospholipids were studied in rats. When administered to rats at a 2% level for 2 wk, both DOA and DEHP caused a hepatomegaly, an increase in hepatic phospholipids and a decrease in the ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE). In the comparable study with mice, the alkyl moiety of DOA was found to be responsible for these alterations. DOA and DEHP specifically altered fatty acid compositions of PC and PE: there was an increase in oleic and palmitic acids and a decrease in stearic and docosahexaenoic acids in PC and an increase in arachidonic acid at the expense of docosahexaenoic acid in PE. In addition, DOA caused an increase in the trienoic and tetraenoic molecular species in PC and an increase in the 1-palmitoyl-2-arachidonyl (16:0//20:4) species in PE. Thus, the effects of DOA on the lipid dynamics resembled those observed with DEHP, although the magnitude was slightly moderated.
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Di-(2-ethylhexyl)phthalate (DEHP) is a chemical additive commonly used in the manufacture of plastic products. The widespread distribution of this compound in the environment and in animal tissues has attracted attention to its possible toxic effect (1). Recently DEHP has been reported to cause hepatic peroxisomal proliferation and hepatic tumors in rats (2,3). Alteration of lipid metabolism (4–8) and inhibition of various enzymes (9,10) in animal models have also been reported. In view of these untoward effects, less toxic substitutes for DEHP have been applied in products such as polyvinylchloride plastic medical appliances. Di-(2-ethylhexyl)adipate (DOA) is one such substitute.

Since DEHP modifies several aspects of lipid metabolism in rats (4–8,11–14) and rabbits (7), it seems important to clarify the effects that DOA exerts on lipid metabolism in the experimental animal model. Bell (15) recently showed that DOA works as an inhibitor of hepatic cholesterogenesis and thus exhibits plasma cholesterol–lowering activity.

The present study was undertaken to examine the effects of DOA on the profiles of hepatic and plasma lipids, and the results were compared with those produced by DEHP. The effects of adipate and 2-ethylhexanol, the constituents of DOA, were also examined.

TABLE 1

Effects of DOA and DEHP on Growth Parameters' Serum and Liver Components and Liver Catalase Activity in Rats (Experiment 1)

Groups	Control	DOA	DEHP
Initial weight (g)	156 ± 3[a]	156 ± 4[a]	156 ± 3[a]
Weight gain (g/2 wk)	74 ± 4[a]	57 ± 4[a]	31 ± 12[c]
Food intake (g/day)	17.7 ± 0.4[a]	16.8 ± 0.8[a]	13.6 ± 0.9[b]
Liver weight (g)	12.3 ± 0.5[a]	13.7 ± 0.6[a]	13.6 ± 0.9[a]
Liver weight (g/100 g body wt)	5.3 ± 0.1[a]	6.5 ± 0.2[b]	7.9 ± 0.2[c]
Liver components (mg/g liver)			
Phospholipids	17.6 ± 0.7[a]	23.0 ± 0.7[b]	26.7 ± 0.9[c]
Triglyceride	5.8 ± 0.3[a]	6.3 ± 0.1[a]	4.8 ± 0.3[b]
Cholesterol	2.1 ± 0.1[a]	2.0 ± 0.1[a]	2.0 ± 0.2[a]
Protein	149 ± 8[a]	179 ± 5[b]	183 ± 7[b]
Glycogen	88 ± 11[a]	54 ± 4[b]	39 ± 5[c]
Catalase activity			
(units/mg protein)	30.7 ± 1.6[a]	59.4 ± 4.6[b]	ND
(units × 10/liver/100 g body wt)	23.3	67.5	ND
Plasma lipids (mg/dl)			
Phospholipids	128 ± 18[a]	106 ± 4[a]	100 ± 9[a]
Triglyceride	143 ± 15[a]	123 ± 7[a]	85 ± 5[b]
Cholesterol	69 ± 2[a]	75 ± 6[a]	62 ± 5[a]

DOA, di-(2-ethylhexyl)adipate; DEHP, di-(2-ethylhexyl)phthalate; ND, not determined. Male rats were fed ad libitum semipurified diets supplemented with either 2% DOA or DEHP for 2 wk. Values are the means ± SE of 5 rats. Values not sharing the same superscript letter within the same row are significantly different at $p < 0.05$.

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Abbreviations: DOA, di-(2-ethylhexyl)adipate; DEHP, di-(2-ethylhexyl)phthalate; PC, phosphatidylcholine; PE, phosphatidylethanolamine; GLC, gas liquid chromatography; TLC, thin layer chromatography.

MATERIALS AND METHODS

Animal experiments. Experiment 1: Six-week-old male Wistar rats (Kyudo Co., Saga, Japan), weighing ca. 156 g, were fed ad libitum the purified basal diet (5) or the basal diet supplemented either with 2% DOA or DEHP. The basal diet consisted (in percent) of vitamin-free casein, 20.0; corn oil, 5.0; cellulose, 4.0; mineral mixture, 4.0; vitamin mixture, 1.0; choline chloride, 0.15 and sucrose to 100. Mineral and vitamin mixtures according to Harper (16) were purchased from Oriental Yeast Co. (Tokyo). DOA and DEHP were from Wako Pure Chemical Industries (Osaka), and the purity was checked by gas liquid chromatography (GLC) with an electron capture detector using a OV-1 column (4); only one peak was detected for both chemicals. Food intake and body weight were measured every day. After 2 wk of feeding, rats were killed by decapitation; livers were excised, rinsed with physiological saline and weighed.

Experiment 2: To assess the effects of the constituents of DOA on liver phospholipid metabolism, 5-week-old ddy mice (Kyudo Co.) weighing ca. 31 g were fed a 20% casein (basal) diet containing either adipate, 2-ethylhexanol (ethylhexanol), a mixture of adipate and ethylhexanol or DOA for 1 wk. The dietary level of these supplements was equivalent to 1% DOA on the molar basis.

Lipid analysis. Liver and serum lipids were extracted and purified by the procedures of Folch et al. (17) and Bligh and Dyer (18), respectively, and were analyzed for phospholipid and triglyceride as reported elsewhere (4,5). Cholesterol was analyzed by the enzymatic method (Cholesterol C-Test, Wako Pure Chemical Industries, Osaka). For separation of individual phospholipids of the liver, total lipid extracts were initially developed on 0.25-mm thick Silica Gel H plates containing 1 mM $NaHCO_3$ under argon in a solvent system of acetone/diethylether (1:3, v/v) to separate neutral lipids from phospholipids, and then were developed in a solvent system of chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v) containing 0.02% butylated hydroxytoluene as an antioxidant. After a brief exposure to iodine vapor, the spots corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol + phosphatidylserine, sphingomyelin, lysoPC and phosphatidic acid were scraped off for the analysis of phosphorus and fatty acid composition (5).

Analysis of molecular species of phospholipids and fatty acid composition. The PC and PE extracted from the plates by the mixture of chloroform/methanol/acetic acid/water (50:39:4:2, v/v/v/v) were hydrolyzed by phospholipase C, and resulting diglycerides were immediately acetylated with acetic anhydrous in pyridine (19,20). The monoacetyldiglycerides were fractionated into molecular species according to the degree of unsaturation by two-step argentation thin layer chromatography (TLC) (19,20). The efficiency of the fractionation was checked by GLC after transmethylation. Pentadecanoic acid was used as an internal standard. Fatty acid compositions of methyl esters from various lipid fractions were determined by GLC on 10% DEGS coated on Chromosorb W (3 mm × 2 m, Gasukuro Kogyo Inc., Tokyo) using a Shimadzu Gas Chromatograph GC-8A. The C-R3A data processor (Shimadzu) was used to data processing. The retention time of each methyl ester was compared with those of the known standards (16:0, 18:0, 18:1, 18:2, 20:4 and 22:6). Based on the relative abundance of molecular species, fatty acid composition of each fraction and the positional distribution of the acyl group, the molecular species compositions were estimated.

Measurement of protein and glycogen. Protein was determined by the method of Lowry et al. (21) using bovine serum albumin as a standard. Liver glycogen was measured as reported previously (5).

Assay of catalase activity. Catalase [EC.1.11.1.6] activity of the liver homogenate was determined spectrometrically (22) at 20 C.

Statistical analysis. Results were shown as the means ± standard error. Comparisons between groups were made by Student's t-test (23).

RESULTS

Effects of DOA and DEHP on growth parameter and liver weight (Experiment 1). Feeding diets containing 2% DOA or DEHP for 2 wk resulted in growth retardation (Table 1). Food intake was also reduced by DEHP but not by DOA. Relative liver weight was increased by feeding plasticizers, in particular DEHP. A marked enlargement of liver by DEHP has been reported previously (4–9).

Effects of DOA and DEHP on the contents of liver lipids, glycogen and protein and on catalase activity (Experiment 1). Feeding DOA resulted in a significant increase in the content of phospholipids and a significant decrease in that of glycogen in the liver (Table 1). The hepatic phospholipids of rats fed DOA increased to 130% of the control value. The increase was attributed mainly to that in PC and PE, which together comprised 75% of the total phospholipids; changes in other components being marginal if any (Table 2). However, the percentage composition of phospholipids was less influenced by the treatment, although the ratio of PC to PE decreased significantly due

TABLE 2

Effects of DOA and DEHP on Hepatic Phospholipid Compositions of Rats (Experiment 1)

Phospholipid	Control (mg/g liver)	DOA (mg/g liver)	DEHP (mg/g liver)
LPC + SPH	0.4 ± 0.0^a (2.4)	0.5 ± 0.1^a (2.2)	0.8 ± 0.1^a (3.0)
PC	9.3 ± 0.3^a (53.0)	11.9 ± 0.2^b (51.7)	12.3 ± 3.5^b (46.3)
PI + PS	2.5 ± 0.1^a (14.1)	2.8 ± 0.1^b (12.2)	3.5 ± 0.1^c (13.0)
PE	4.1 ± 0.1^a (23.4)	6.1 ± 0.3^b (26.4)	8.3 ± 0.5^c (30.9)
PA	1.3 ± 0.1^a (7.2)	1.7 ± 0.1^b (7.5)	1.8 ± 0.1^b (6.9)

Phospholipids were separated by thin layer chromatography and determined as described in Materials and Methods. Values are the means ± SE of 5 rats. Values not sharing the same superscript letter within the same row are significantly different at $p < 0.05$. DOA, di-(2-ethylhexyl)adipate; DEHP, di-(2-ethylhexyl)phthalate; PC, phosphatidylcholine; LPC, lysoPC; SPH, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PA, phosphatidic acid.

TABLE 3

Effects of DOA and DEHP on the Fatty Acid Compositions of Liver Phosphatidylcholine and Phosphatidylethanolamine of Rats (Experiment 1)

Fatty acid	Control	DOA	DEHP
Phosphatidylcholine			
16:0	28.0 ± 1.7^{a}	33.6 ± 1.0^{b}	36.1 ± 1.3^{b}
16:1	2.7 ± 0.5^{a}	3.2 ± 0.1^{a}	tr
18:0	19.8 ± 1.2^{a}	15.2 ± 0.6^{b}	12.8 ± 1.3^{b}
18:1	8.5 ± 0.4^{a}	10.2 ± 0.3^{b}	15.1 ± 0.5^{c}
18:2	11.6 ± 0.6^{a}	11.5 ± 1.1^{a}	12.1 ± 0.5^{a}
20:4	23.0 ± 1.3^{a}	22.9 ± 1.4^{a}	21.0 ± 1.3^{a}
22:6	6.2 ± 0.4^{a}	3.3 ± 0.3^{b}	2.0 ± 0.2^{c}
Phosphatidylethanolamine			
16:0	23.6 ± 1.7^{a}	24.2 ± 2.2^{a}	17.3 ± 0.7^{b}
16:1	tr	tr	tr
18:0	23.4 ± 1.3^{a}	27.2 ± 1.3^{a}	26.1 ± 1.1^{a}
18:1	6.8 ± 0.4^{a}	6.3 ±0.3^{a}	7.6 ± 0.7^{a}
18:2	4.1 ± 0.3^{a}	4.1 ± 0.3^{a}	4.9 ± 0.2^{a}
20:4	26.1 ± 0.9^{a}	28.7 ± 1.3^{a}	36.0 ± 0.5^{a}
22:6	15.8 ± 1.2^{a}	9.5 ± 1.0^{b}	8.1 ± 0.5^{b}

See Tables 1 and 2. DOA, di-(2-ethylhexyl)adipate; DEHP, di-(2-ethylhexyl)phthalate; tr, trace. Liver phosphatidylcholine and phosphatidylethanolamine were separated as described in Materials and Methods. Minor fatty acids (less than 1% of the total fatty acids detected) were excluded from the table. Values are the means ± SE of 5 rats. Values not sharing the same superscript letter within the same row are significantly different at $p < 0.05$.

TABLE 4

Effects of DOA and DEHP on the Molar Relative Abundance of Liver Phosphatidylcholine and Phosphatidylethanolamine of Rats (Experiment 1)

Molecular classes	Control	DOA	DEHP
Phosphatidylcholine			
Saturated	3.7 ± 0.3^{a}	1.8 ± 0.1^{b}	1.5 ± 0.2^{c}
Monoenoic	13.4 ± 1.8^{a}	16.0 ± 0.3^{a}	22.8 ± 0.4^{b}
Dienoic	17.0 ± 0.4^{a}	16.6 ± 0.4^{a}	16.4 ± 0.3^{a}
Trienoic	3.2 ± 0.2^{a}	6.2 ± 0.2^{b}	6.6 ± 0.2^{b}
Tetraenoic	47.0 ± 0.1^{a}	50.1 ± 0.6^{b}	49.2 ± 0.5^{b}
Hexaenoic	14.6 ± 1.5^{a}	10.4 ± 0.5^{b}	7.0 ± 0.4^{c}
Phosphatidylethanolamine			
Saturated	0.5 ± 0.1^{a}	0.5 ± 0.2^{a}	0.4 ± 0.0^{a}
Monoenoic	2.2 ± 0.5^{a}	3.2 ± 0.3^{a,b}	3.6 ± 0.3^{b}
Dienoic	7.1 ± 0.3^{a}	6.3 ± 0.9^{a}	6.4 ± 0.4^{a}
Trienoic	2.3 ± 0.3^{a}	3.2 ± 0.1^{a}	2.4 ± 0.3^{a}
Tetraenoic	51.4 ± 1.9^{a}	58.3 ± 2.0^{b}	66.5 ± 1.6^{c}
Hexaenoic	33.4 ± 1.7^{a}	24.3 ± 1.9^{b}	16.5 ± 1.1^{c}

See Tables 1 to 3. DOA, di-(2-ethylhexyl)adipate; DEHP, di-(2-ethylhexyl)phthalate. Each molecular class was isolated by argentation thin layer chromatography, and the percentage concentration was determined by the internal standard (pentadecanoic acid) method. Values are the means ± SE (n = 4–5). Values not sharing the same superscript letter within the same row are significantly different at $p < 0.05$.

to a reciprocal change in these phospholipids. A similar but more profound response was observed in rats given DEHP, consistent with reported data (5,6,12). No demonstrable difference due to DOA was observed in the hepatic triglyceride level. The plasticizers caused a slight but significant increase in the concentration of liver protein. Hepatic catalase activity in rats fed DOA increased, suggesting an induction of peroxisomes. The induction of this enzyme has already been reported for DEHP, monoethylhexylphthalate and ethylhexanol (14,24).

Effects of DOA and DEHP on plasma lipids (Experiment 1). The plasma concentrations of phospholipids and triglyceride tended to decrease after feeding DEHP or DOA compared with the controls (Table 1). The magnitude of the decrease was more prominent in the DEHP than in the DOA groups. There was no significant difference in the cholesterol level among the three groups.

Effects of DOA and DEHP on the fatty acid composition of liver PC and PE (Experiment 1). Table 3 summarizes the effects of DOA and DEHP on the fatty acid compositions of hepatic PC and PE. Administration of DOA and DEHP altered the fatty acid compositions of these phospholipids, in particular in the latter; in the PC fraction, the proportion of oleic and palmitic acids increased at the expense of stearic and docosahexaenoic acids. In the PE fraction, DEHP caused an increase in the proportion of arachidonic acid and a decrease in palmitic and docosahexaenoic acids. The DOA effect on PE was less clear, and decreased proportion of docosahexaenoic acid was the sole variable. A similar change in the fatty acid composition of phospholipids has also been observed in the hepatic microsomes and mitochondria (Yanagita, T., unpublished data).

Effects of DOA and DEHP on the molecular species of liver PC and PE (Experiment 1). Table 4 gives the proportion of molecular species of hepatic PC and PE. Six different groups of acetyldiacylglycerol derivatives with different degrees of unsaturation were obtained, i.e. saturated, monoenoic, dienoic, trienoic, tetraenoic and hexaenoic species. The fatty acid profiles indicate that the most striking change induced by DEHP in the molecular classes of PC was an increase in the monoenoic and trienoic classes and a decrease in hexaenoic classes, whereas in PE the monoenoic and tetraenoic classes increased and the hexaenoic class decreased. DOA also altered the molecular species compositions of these phospholipids similarly, but to a lesser extent.

Based on the relative abundance of molecular species (Table 4), the fatty acid composition of each fraction and the positional distribution of the acyl group, the molecular species composition was estimated (Tables 5 and 6). Fifteen major (more than 2% of total PC and PE populations) molecular species were found. The relative abundance of the molecular species of PC in the liver of the control rats was comparable with that reported by other workers (19,25).

The DEHP treatment in general resulted in an increase in the PC molecular species containing palmitic acid and a decrease in those containing stearic acid. In addition, there was a predictable decrease in the molecular species containing docosahexaenoic acid, irrespective of the partner fatty acids, palmitic or stearic acids.

Alteration of the molecular species composition of PC by DOA was comparable with those produced by DEHP, although the magnitude of alteration was considerably low.

Both plasticizers also induced the change in the molecular species composition of hepatic PE (Table 6). A marked

change by the DEHP treatment was an increased proportion of the tetraenoic species, particularly the (18:0//20:4) PE and the decreased proportion of hexaenoic species, particularly the (16:0//22:6) PE. Thus, DEHP altered the (18:0//20:4) species in the PC and PE fractions in a different manner, a decrease in the former and an increase in the latter. The effect of DOA on the PE species was rather moderate and was again most obvious in the hexaenoic species.

Effects of adipate and ethylhexanol on liver phospholipids (Experiment 2). There were no significant differences in body weight gain and food intake in all groups. The increase in the concentration of hepatic phospholipids by DOA was again detected in mice. The increased concentration of hepatic phospholipids was also found in mice fed either ethylhexanol or a mixture of adipate and ethylhexanol, whereas the effect of adipate did not appear (Table 7). These results thus indicate that the alkyl moiety of the adipate ester is responsible for the effect of DOA. A similar result has been obtained in the corresponding study with DEHP (6).

DISCUSSION

In the present study, both DOA and DEHP induced quantitative and qualitative alterations of the hepatic phospholipids: an increase in the concentration of phospholipids and a change in the proportion of individual phospholipids. The modification of the phospholipid pattern by DEHP corresponded to that reported for liver microsomes and mitochondria (5). The alteration of the PC to PE ratio induced by DOA and DEHP is at least interpreted as the results of the increased synthesis of PC and PE from diglyceride accompanying the decreased conversion of PE to PC. In fact, the incorporation of [^{3}H]glycerol in vivo and in vitro and of [^{14}C]acetate in vitro into liver phospholipids increased after feeding DEHP or DOA (Yanagita, T., et al., in preparation). The activities of glycerol-3-phosphate acyltransferase and CTP:phosphocholine cytidyltransferase, the rate-limiting enzymes of glycerophospholipid biosynthesis (25–28), in liver microsomes were also simultaneously induced in rats treated with DEHP (39). The possibility of the decreased conversion of PE to PC remains to be confirmed.

Both plasticizers also caused an alteration of the proportion of palmitate/stearate and oleate/stearate of phospholipids. It is likely that DOA and DEHP could induce the stimulation of the fatty acid synthesis (13), although this is controversial (7,15). DEHP also presumably increases the activity of stearoyl-CoA desaturase of liver microsomes (14), thus resulting in the increase in the percentage of oleic acid and the decrease in that of stearic acid (Table 3). Other characteristic changes in the fatty acid compositions were an increase in arachidonic acid in PE from rats fed DEHP and a decrease in docosahexaenoic acid in PC and PE from rats fed DEHP or DOA.

The fatty acid composition indicates that these plasticizers caused the alteration of the profile of the molecular species of liver PC and PE (Tables 5 and 6). When an increase in the concentration of PC and PE is taken into account, the changes in the species of the (16:0//18:1) PC, the (16:0//18:2) PC, the (16:0//20:4) PC and the (16:0//20:4) PE were further accentuated. Alternatively, a change in the proportion of the (18:0//20:4) PC and the (18:0//18:2) PC disappeared.

For formation of the monoenoic and dienoic species of liver PC and PE, a de novo synthesis appears responsible (26), while the tetraenoic species are synthesized mainly from 1-acyl-lyso derivatives (29,30). The hexaenoic species of hepatic PC appears to be derived mainly from PE by successive methylation (31,32). In fact, the changes caused by the plasticizers are related to both the enhance-

TABLE 5

Effects of DOA and DEHP on the Molecular Association of Fatty Acids in Liver Phosphatidylcholine of Rats (Experiment 1)

Fatty acids		Control	DOA	DEHP
Pos. 1	Pos. 2	(mol %)	(mol %)	(mol %)
Disaturated		3.2 ± 0.3^a	2.1 ± 0.2^a	1.9 ± 0.1^b
16:0	16:1	1.5 ± 0.0^a	1.4 ± 0.1^a	0.2 ± 0.0^b
16:0	18:1	10.3 ± 1.2^a	12.6 ± 0.6^a	19.2 ± 0.1^b
18:0	18:1	1.9 ± 0.4^a	1.8 ± 0.2^a	2.4 ± 0.1^a
16:0	18:2	9.4 ± 0.5^a	11.0 ± 0.5^a	10.8 ± 0.4^a
18:0	18:2	7.2 ± 0.4^a	5.6 ± 0.2^b	5.2 ± 0.1^b
16:0	20:3	0.7 ± 0.1^a	1.9 ± 0.2^b	2.6 ± 0.2^b
18:0	20:3	0.8 ± 0.1^a	1.3 ± 0.1^b	1.6 ± 0.1^c
18:1	18:2	2.7 ± 0.3^a	3.3 ± 0.2^a	2.9 ± 0.2^a
16:0	20:4	20.6 ± 0.2^a	27.1 ± 0.4^b	28.0 ± 0.3^b
18:0	20:4	21.5 ± 0.3^a	18.0 ± 0.7^b	16.2 ± 0.2^c
18:1	20:3	1.1 ± 0.1^a	1.6 ± 0.3^a	1.1 ± 0.2^a
16:0	22:6	9.5 ± 0.0^a	6.1 ± 0.3^b	3.9 ± 0.2^c
18:0	22:6	6.0 ± 0.1^a	2.9 ± 0.2^b	2.0 ± 0.1^c

See Tables 3 and 4. DOA, di-(2-ethylhexyl)adipate; DEHP, di(2-ethylhexyl)phthalate. The molecular species of liver phosphatidylcholine was calculated as described in Materials and Methods. Values are the means ± SE of 5 separate determinants from 5 rats. Values not sharing the same superscript letter within the same row are significantly different at $p < 0.01$.

TABLE 6

Effects of DOA and DEHP on the Molecular Association of Fatty Acids in Liver Phosphatidylethanolamine of Rats (Experiment 1)

Fatty acids		Control	DOA	DEHP
Pos. 1	Pos. 2	(mol %)	(mol %)	(mol %)
Disaturated		0.5 ± 0.2^a	0.2 ± 0.3^a	0.2 ± 0.1^a
16:0	18:1	0.3 ± 0.0^a	0.2 ± 0.1^a	0.1 ± 0.0^a
18:0	18:1	0.7 ± 0.1^a	1.5 ± 0.1^b	1.5 ± 0.1^b
16:0	18:2	3.8 ± 0.6^a	4.0 ± 0.2^a	2.9 ± 0.2^b
18:0	18:2	3.7 ± 0.5^a	4.0 ± 0.1^a	3.7 ± 0.3^a
18:1	18:2	3.1 ± 0.3^a	1.9 ± 0.4^a	2.5 ± 0.5^a
16:0	20:4	15.2 ± 0.2^a	18.0 ± 0.6^b	19.0 ± 0.5^b
18:0	20:4	31.0 ± 0.9^a	32.9 ± 1.1^a	38.2 ± 1.2^b
18:1	20:4	5.8 ± 1.5^a	7.8 ± 0.6^a	7.8 ± 1.2^a
16:0	22:6	19.6 ± 0.6^a	14.5 ± 0.9^b	9.2 ± 0.2^c
18:0	22:6	11.8 ± 0.6^a	9.1 ± 0.4^b	8.6 ± 0.3^b

See Tables 3 and 4. DOA, di-(2-ethylhexyl)adipate; DEHP, di-(2-ethylhexyl)phthalate. The molecular species composition of liver phosphatidylethanolamine was calculated as described in Materials and Methods. Values are the means ± SE of 5 separate determinants from 5 rats. Values not sharing the same superscript letter within the same row are significantly different at $p < 0.01$.

TABLE 7

Effects of DOA and Its Components on Liver Weight and Liver Phospholipids in Mice (Experiment 2)

Groups	Body weight, initial gain (g)	Body weight, initial gain (g/7 days)	Food intake (g/day)	Relative liver weight (g/100 g body weight)	Liver phospholipids (mg/g)	Liver phospholipids (mg/relative liver weight)
Control	31 ± 1[a]	3 ± 1[a]	5.1 ± 0.7[a]	4.6 ± 0.14[a]	26.7 ± 1.1[a]	123 ± 5[a]
DOA	31 ± 1[a]	1 ± 1[a]	4.2 ± 0.5[a]	7.1 ± 0.32[a]	30.4 ± 0.6[b]	215 ± 5[b]
Adipate	31 ± 1[a]	2 ± 1[a]	4.8 ± 0.3[a]	4.8 ± 0.03[a]	26.8 ± 0.3[a]	128 ± 3[a]
Ethylhexanol	31 ± 0[a]	0 ± 1[a]	4.0 ± 0.5[a]	6.4 ± 0.11[b]	32.1 ± 0.9[b]	205 ± 7[b]
Adipate + ethylhexanol	31 ± 1[a]	1 ± 1[a]	4.3 ± 0.4[a]	6.0 ± 0.19[b]	33.9 ± 0.9[b]	203 ± 6[b]

DOA, di-(2-ethylhexyl)adipate. Male mice were fed each diet for 1 week. Each chemical added to the basal diet was adjusted to be quivalent to 1% DOA on the molecular basis. Values are the means ± SE of 6 mice. Values not sharing the same superscript letter within the same column are significantly different at $p < 0.05$.

ment of de novo synthesis (39) and the induction of microsomal 1-acylglycerophosphorylcholine acyltransferase (33). Thus, the observed alterations of hepatic phospholipids could result from the complex interplay of a number of enzymatic systems involved in phospholipid synthesis and the pool size and the metabolic turnover of the individual components.

Phospholipids are the integral components of the biological membranes and influence the function of the membranes, as shown by their role in the hepatic microsomal drug-metabolizing systems (34) or in the activities of microsomal UDP-glucuronyltransferase and glucose-6-phosphatase (35,36). However, we have found that mannose-6-phosphatase, an enzyme located to the lumenal face of microsomal membranes, is highly latent in liver microsomes from both DEHP-fed and control rats, due to a hindrance of the substrate to the site of hydrolysis (37). An asymmetric distribution of phospholipids in liver microsomal membranes with PC predominantly in the cytoplasmic face and a slight excess of PE in the lumenal face appears to remain apparently unchanged, even after treatment with DEHP (39).

In conclusion, the present study showed that DOA or DEHP induced the quantitative and qualitative alteration of hepatic phospholipids. These changes were less prominent in rats treated with DOA than in those treated with DEHP. The difference in the hydrolytic rate and/or the diverse effect of metabolites of DOA and DEHP (38) might imply these differences. The effect of DOA was essentially attributable to its alkyl moiety, ethylhexanol but not adipate as in the case of DEHP (6).

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Derivatization of Keto Fatty Acids, X: Synthesis of Thiazanones

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The synthesis of alkyl chain-substituted thiazanones from oxo fatty esters and a long chain aldehyde is reported. Four oxo compounds—methyl 10-oxoundecanoate, methyl 9-oxooctadecanoate, methyl 9,10-dioxooctadecanoate and octadecanal—were allowed to react with β-mercaptopropionic acid in the presence of ammonium carbonate in benzene to give the corresponding 4-*m*-thiazanones in high yields. The structures of these compounds were confirmed by combustion and spectral data.
Lipids 22, 578-582 (1987).

Thiazanones are six-membered heterocyclic compounds containing one nitrogen and one sulphur atom with a carbonyl function. Fatty acids possessing a chain-substituted thiazanone ring have not yet been reported in the literature. Only a few methods are available for the synthesis of thiazanones: 2-amino(imino)-4-oxo-5,6-dihydro-1,3-thiazinium bromide was prepared by heating β-bromopropionyl chloride [$Br(CH_2)_2COCl$] and ammonium thiocyanate followed by reaction with ammonia (1), 2-*p*-chlorophenyl-3-alkyl-4-*m*-thiazanones were obtained by treatment of mercaptals with *p*-chlorobenzaldehyde (2), and benzothiazinones were synthesized by condensation of *o*-mercaptobenzamide with methyl acetylenedicarboxylate (3). A number of 1,3- and 1,4-benzothiazinones have been evaluated for their central nervous system (CNS) depressant activity (4). Incorporation of the thiazanone ring in fatty acid chain is expected to enhance the application of these derivatives. Recently, we have reported the synthesis of fatty chain-substituted 4-thiazolidinones (5). In continuation of our work on derivatization of keto fatty acids (6), we synthesized chain-substituted thiazanone derivatives by condensing keto fatty esters and a long chain aldehyde with β-mercaptopropionic acid in the presence of ammonium carbonate.

Long chain oxo compounds, having a carbonyl function at penultimate (methyl 10-oxoundecanoate, I), internal (methyl 9-oxooctadecanoate, II), vicinal dioxo (9,10-dioxooctadecanoate, III) and terminal (1-octadecanal, IV) positions were used for the synthesis of thiazanone derivatives.

MATERIALS AND METHODS

All mp were observed on Koffler apparatus and were uncorrected. The spectroscopic and chromatographic methods were the same as detailed in our previous paper (5) except where specified. Lithium aluminium hydride ($LiAlH_4$, >98%), chromic anhydride, methylene chloride and pyridine were supplied by E. Merck (Darmstadt, FRG) and β-mercaptopropionic acid by Sigma Chemical Co. (St. Louis, MO). The methyl esters of long chain oxo acids were prepared by refluxing them with absolute methanol and a few drops of sulphuric acid. The abbreviations s, t, m, and br denote singlet, triplet, multiplet and broad, respectively. Petroleum ether refers to a fraction of bp 40-60 C, and ether refers to diethyl ether.

Preparation of oxo fatty acids (I-III). 10-Oxoundecanoic (I, mp 58-59 C) and 9,10-dioxooctadecanoic (III, mp 86 C) acids were prepared according to the methods discussed in an earlier paper (5). 9-Oxooctadecanoic acid (II) was obtained from pure *Wrightia tinctoria* seed oil (7) using Gunstone's partitioning procedure (8). 9-Hydroxy-12-octadecenoic acid isolated from this seed oil was hydrogenated by palladium on charcoal in ethyl acetate to give 9-hydroxyoctadecanoic acid (mp 80-81 C) which, on Jones' oxidation (10), afforded 9-oxooctadecanoic acid [mp 79-80 C; lit. (8) 79.5 C]. IR (CCl_4): 1710 (acid carbonyl), 1720 cm^{-1} (chain carbonyl).

Preparation of octadecanal (IV). Methyl stearate (10 g) was dissolved in dry ether (500 ml) and slowly added to a stirred suspension of $LiAlH_4$ (2 g) in dry ether (200 ml). After stirring for a further 10 min at room temperature, excess of $LiAlH_4$ was destroyed by the cautious addition of wet ether and then water. Dilute H_2SO_4 (2 l, 2M) was subsequently added and the octadecanol was isolated. On crystallization from petroleum ether this gave a white solid in 90% yield mp 58-58.5 C [lit. (11), 59 C].

Octadecanol was oxidized to octadecanal by chromic anhydride (12). To a stirred mixture of pyridine (9.65 ml, 0.12 mol) and methylene chloride (150 ml), chromic anhydride (6 g, 0.06 mol) was added, and the temperature was kept below 25 C using an ice bath. After 15 min, the mixture cleared to a dark red solution. Octadecanol (2.6 g, 0.01 mol) in methylene chloride (15 ml) was added in portions, and the mixture turned dark brown. After 30 min, the solvent was removed in vacuo, and the residue was extracted with ether. The ethereal layer was washed successively with 10% aqueous NaOH, 10% HCl, sat. $NaHCO_3$ solution and water and dried to give 98% octadecanal (IV) mp 37-38 C [lit. (11) 38 C]. IR (CCl_4): 2715 (aldehydic C-H stretch), 1735 cm^{-1} (CHO); ^1H NMR ($CDCl_3$): δ 0.88 (3, t, CH_3), 1.3 (30, br s, chain methylene), 3.6 (2, m, CH_2CHO), 9.6 (1, br m, CHO).

Conversion of oxo fatty esters to thiazanones. The thiazanones (Ia-IVa) were synthesized from their corresponding oxo fatty esters (I-IV). A mixture of dry benzene (50 ml) and β-mercaptopropionic acid (6.45 g) was refluxed in a round-bottom flask using a Dean Stark separator for 3 hr. Excess of β-mercaptopropionic acid was used to obtain IIIa from III. Oxo fatty acid ester (1.0 g) and ammonium carbonate (11.0 g) were added to the cooled mixture (5). A vigorous reaction took place. The contents were refluxed, and the progress of reaction was monitored by thin layer chromatography (TLC). At the end of the reaction, the solvent was removed under reduced pressure. The residue was extracted with ether, washed with 10% $NaHCO_3$ solution and dried over anhydrous sodium sulphate. The product thus obtained was crystallized from petroleum ether/benzene (4:1, v/v).

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TABLE 1

Experimental and Spectral Data of Thiazanones

Thiazanone	Rxn time (hr)	Yield (%)	m.p. (°C)	IR (Cm^{-1})	^1H NMR in $CDCl_3$ (δ,ppm)	Combustion analysis (%)
Ia	24	48	53	(CCl_4): 3170,1735,1660, 1405,1225,720,670	1.30,1.55,1.75,2.30, 2.55,2.85,3.68,8.60	calcd. for $C_{15}H_{27}O_3NS$: C,59.81;H,9.04;N,4.65 found:C,59.79;H,8.96;N,4.70
IIa	30	86	—	(film):3170,1730,1665, 1225,710,690,1410	0.88,1.30,1.65,2.30, 2.60,2.82,3.65,8.50	calcd. for $C_{22}H_{41}O_3NS$: C,66.06;H,10.33;N,3.50 found:C,66.15;H,10.27;N,3.48
IIIa	50	96	—	(film):3185,1740,1715, 1660,1405,1230,770,640	0.88,1.30,1.65,2.27 2.57,2.88,3.70,8.40	calcd. for $C_{22}H_{39}O_4NS$: C,63.89;H,9.51;N,3.39 found:C,63.92;H,9.49;N,3.38
IVa	10	91	67	(CCl_4):3160,1670,1410, 1230,780,690	0.88,1.30,1.80,2.53, 2.82,4.52,8.50	calcd. for $C_{21}H_{41}ONS$: C,70.80;H,11.61;N,3.94 found:C,70.90;H,11.59;N,3.90

The experimental and spectral data of respective thiazanones are summarized in Table 1.

RESULTS AND DISCUSSION

The oxo compounds (I–IV) on treatment with β-mercaptopropionic acid in the presence of ammonium carbonate following the procedure of Paryzek et al. (13) yielded the corresponding 4-*m*-thiazanones (Ia–IVa) as shown in Scheme 1.

The IR spectrum of Ia (Table 1) revealed characteristic bands at 3170 (NH stretching), 1735 and 1660 (ester and lactam carbonyl), 1405 (C-N stretch), 1225 (C-S wagging), 720 (NH wag.) and 670 cm^{-1} (C-S stretch). These values are in agreement with the structure of Ia. The ^1H NMR spectrum of Ia exhibited two sharp singlets at δ 1.55 for terminal ω-methyl protons and at δ 3.68 for ester methyl protons. The ring protons gave triplet signals at δ 2.85 (CH_2-S) and 2.55 (CH_2CONH) while NH proton appeared at δ 8.6 as a broad singlet. The methylene protons alpha to ring and ester carbonyl appeared at δ 1.75 and 2.30, respectively. The chain methylene protons absorbed at δ 1.30 as a broad singlet. On the basis of these data, the product Ia was formulated as 2-8′-carbomethoxyoctyl-2-methyl-4-*m*-thiazanone.

Thiazanone IIa gave similar IR and ^1H NMR characteristics as Ia except for the appearance of terminal methyl and four methylene protons (α- to ring) at δ 0.88 and 1.65, respectively. The product IIa was characterized as 2-7′-carbomethoxyheptyl-2-nonyl-4-*m*-thiazanone.

Similarly, the thiazanone (IVa) located at a terminal position displayed almost the same diagnostic peaks in IR and ^1H NMR spectra as observed in Ia and IIa. The only characteristic ^1H NMR peak observed was at δ 4.52 for the methine proton attached to C-2 position of the ring. The product IVa was identified as 2-heptadecyl-4-*m*-thiazanone.

The thiazanone (IIIa) obtained from the vicinal dioxo ester (III) exhibited a clear single spot on the TLC plate. It showed IR absorption bands at 3185 (NH stretch), 1740 and 1660 (ester and lactam carbonyl), 1405 (C-N stretch), 770 (NH Wag.) 1230 and 640 (C-S wag. and stretch), along with a free carbonyl band at 1715 cm^{-1}. The ^1H NMR spectrum exhibited a multiplet at δ 2.27 integrating for four methylene protons α- to chain and ester carbonyls. The two methylene protons alpha to the ring appeared at δ 1.65 along with other usual peaks observed for IIa. These data clearly confirmed that one of the two carbonyl groups of IIIa has not taken part in the reaction even on prolongation of reaction time and use of excess of the reagent. On the basis of these data, product IIIa was

I, II, IV → Ia, IIa, IVa (reagents (i), (ii))

I, Ia : R = CH_3 ; R′ = $(CH_2)_8COOCH_3$
II, IIa : R = $CH_3(CH_2)_8$; R′ = $(CH_2)_7COOCH_3$
IV, IVa : R = $CH_3(CH_2)_{16}$; R′ = H

III → IIIa (reagents (i), (ii))

III, IIIa : R = $CH_3(CH_2)_7$; R′ = $(CH_2)_7COOCH_3$
(i)= $SHCH_2CH_2COOH$, (ii) = $(NH_4)_2CO_3$ / benzene

SCHEME 1. Synthesis of fatty thiazanones.

characterized as an isomeric mixture of 2-7′-carbomethoxyheptyl-2-1′-oxononyl-4-*m*-thiazanone/2-(8′-carbomethoxyl-1′-oxooctyl)-2-octyl-4-*m*-thiazanone. From the foregoing discussion, it can be inferred that only one carbonyl group participates in the reaction. Since both the carbonyl functions are in the middle of the chain, an isomeric mixture of products was expected that could not be resolved by chromatographic methods. It appeared that steric hindrance (5) caused by the introduction of one thiazanone ring made the adjacent carbonyl function unable to undergo further attack by the reagent.

Mass fragmentation of thiazanones. Very few reports are available for the detailed mass spectral studies of thiazanones. Russian workers (14) have studied the mass fragmentation of 2-anilino-5,6-dihydro-4H-1,3-thiazin-5-one and observed that the cleavage mainly occurs at the C-N bond. We also recently studied the basic fragmentation of thiazolidinones derivatives of fatty acids (5). We now discuss the mass spectra (MS) of thiazanones (Ia–IVa), which were found not to be much different from thiazolidinones (5) except for one more methylene group in the ring. The compositions of fragment ions were supported by accurate mass measurement studies. The composition and relative intensity of fragment ions are indicated in parentheses.

MS of Ia (Fig. 1): m/z 301 (M^+,$C_{15}H_{27}O_3NS$, 59.6), 286 (M-CH_3, 26.8), 270 (M-OCH_3, 98.3) 268 (M-SH, 37.0), 229 (M-$CH_2CH_2CONH_2$, 9.8), 214 (M-$COCH_2CHS$, 99.1), 212 (M-$COCH_2CH_2SH$, 99.1), 196 (M-C_3H_7ONS, 98.7), 183 (214-OCH_3, 49.5), 182 (214-CH_3OH, 95.0), 158 (M-$C_8H_{15}O_2$, 57.0), 131 (A+1, 97.0), 130 (A, base peak), 112 (A-CO, 59.8) and 89 ($COCH_2CH_2SH$, 44.7).

Ia

MS of IIa (Fig. 2): m/z 399 (M^+, $C_{22}H_{41}O_3NS$, 6.7), 368 (M-OCH_3, 7.0), 366 (M-SH, 4.7), 351 (M-CH_3SH, 5.6), 338 (M-C_2H_5S, 6.5), 327 (M-$CH_2CH_2CONH_2$, 10.7), 324 (M-$COCH_2SH$, 3.8), 312 (M-$COCH_2CHS$, 35.2), 310 (M-$COCH_2CH_2SH$, 8.6), 295 (M-C_3H_6NOS, 4.6), 272 (C, 9.0), 242 (B, 10.5), 240 (C-CH_3OH, 4.0), 239 (C-SH, 4.8), 200 (B-CH_2CO, 65.7), 185 (B-$NHCOCH_2$, 29.5), 170 (B-$NH_2COCH_2CH_2$, 52.3), 71 (C_3H_5ON, 80.0) and 55 (C_3H_3O, base peak).

IIa

MS of IIIa (Fig. 3): m/z 413 (M^+, $C_{22}H_{39}O_4NS$, 10.0), 382 (M-OCH_3, 5.4), 380 (M-SH, 3.9), 353 (M-CH_2CH_2S, 2.2), 341 (M-$CH_2CH_2CONH_2$, 9.7), 326 (M-$COCH_2CHS$, 3.6), 324 (M-$COCH_2CH_2SH$, 9.0), 315 (McLafferty, 4.5), 309 (M-C_3H_6ONS, 4.3), 300 (M-C_8H_{17}, 8.5), 273 (D+H, 11.7), 272 (D, 35.1), 271 (McLafferty, 80.1), 240 (D-32, 34.2), 229 (F+H, 3.7), 228 (F, base peak), 185 (G, 21.6), 153 (G-32, 3.9) and 141 (E, 23.4).

IIIa

FIG. 1. Mass spectrum of 2-8′-carbomethoxyoctyl-2-methyl-4-*m*-thiazanone (Ia).

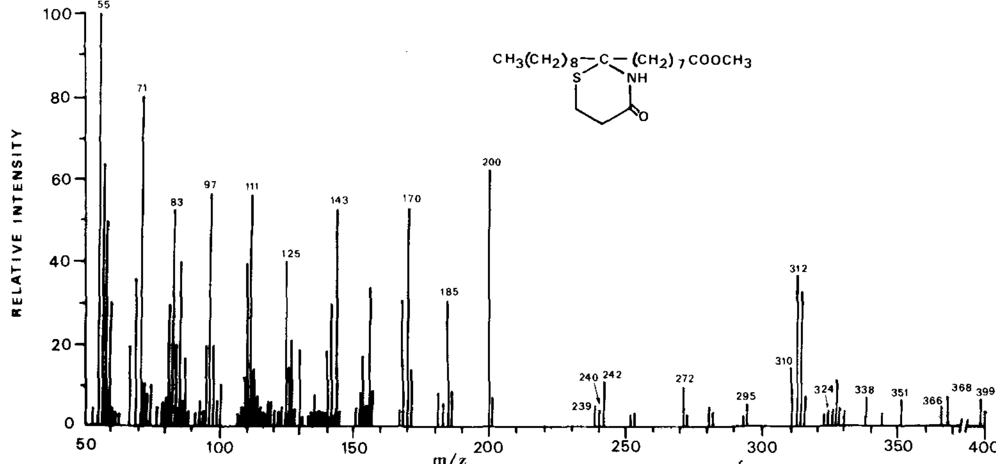

FIG. 2. Mass spectrum of 2-7′-carbomethoxyheptyl-2-nonyl-4-*m*-thiazanone (IIa).

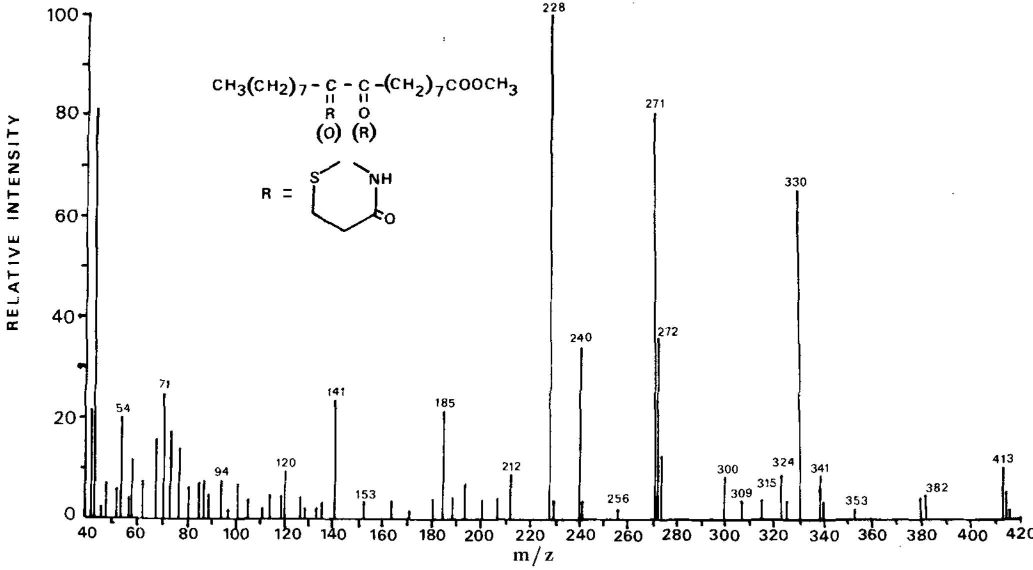

FIG. 3. Mass spectrum of 2-7′-carbomethoxyheptyl-2-1′oxononyl-4-*m*-thiazanone/2(8′-carbomethoxyl-1′-oxo-octyl)-2-octyl-4-*m*-thiazanone (IIIa).

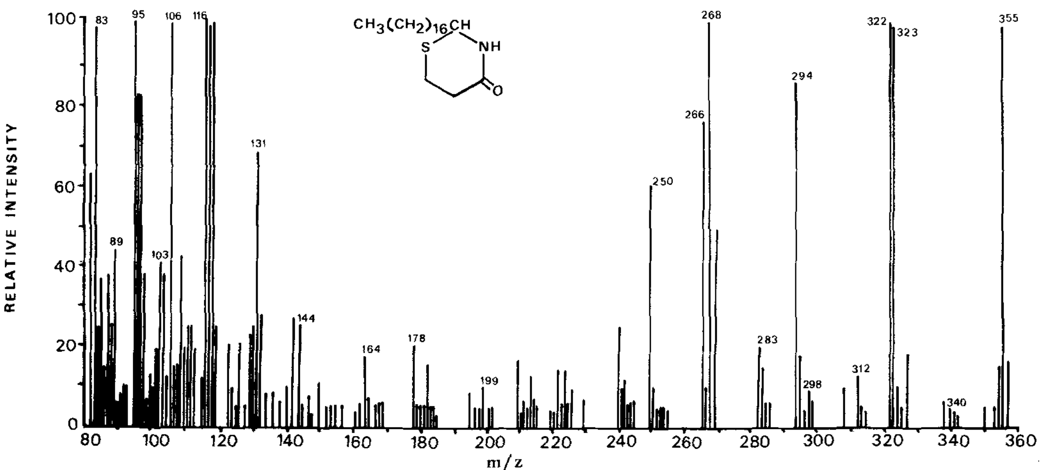

FIG. 4. Mass spectrum of 2-heptadecyl-4-*m*-thiazanone (IVa).

MS of IVa (Fig. 4): m/z 355 (M^+, $C_{21}H_{41}ONS$, 99.0), 340 (M-NH, 5.1), 323 (M-32, 99.1), 322 (M-SH, 99.5), 312 (M-NHCO, 12.2), 298 (M-$NHCOCH_2$, 9.1), 294 (M-C_2H_5S, 84.6), 283 (M-$CH_2CH_2CONH_2$, 20.4), 268 (M-$COCH_2CHS$, 98.1), 266 (M-$COCH_2CH_2SH$, 75.5), 251 (M-C_3H_6ONS, 10.2), 117 (M-$C_{17}H_{34}$, 98.8), 116 (H, base peak), 103 (C_3H_4ONS, 37.7) and 89 (C_3H_5OS, 42.8).

IVa

These MS studies revealed that cleavage alpha to the heterocyclic ring and toward the ester carbonyl gave the base peak in the case of Ia and IIIa. The compound IVa gave a base peak by alpha cleavage on the alkyl chain side of the ring. M-$COCH_2CH_2SH$ appears to be the diagnostic peak of thiazanones, which arises by the cleavages of the 1,2 and 3,4 bonds. The cleavages at the 2,3 and 1,6 bonds afforded another characteristic peak, M-$CH_2CH_2CONH_2$, of lower intensity. These two peaks are triggered by the loss of two methylene groups, a carbonyl function together with either SH or NH_2 from the molecular ion and thus confirm the structure of the thiazanone ring. Other peaks of interest were M-SH and M-$COCH_2CHS$.

The thiazanones synthesized here may be evaluated for CNS depressant effect. The lipophilization of thiazanone ring may enhance their activity.

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Effect of Cold Environment on Hepatic Microsomal Δ6 and Δ9 Desaturase Activity of Male Rats

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Male rats maintained at 24 C and then shifted to 5 C for 5 days increased food intake and decreased in growth rate and food conversion. No modification was observed in Δ6 desaturase activity, while Δ9 desaturase activity decreased after this period of time. These results were confirmed by liver microsomal and mitochondrial fatty acid composition. The phospholipid composition of liver microsomes was unaltered, whereas in mitochondria, phosphatidylcholine and sphingomyelin decreased and phosphatidylethanolamine increased due to the cold environment. The influence of food intake and weight changes on fatty acid metabolism was studied using (i) rats maintained at 5 C with restricted food intake to match the food intake of those kept at 24 C with food ad libitum and (ii) rats maintained at 24 C whose food intake was also restricted so that their growth rate would be the same as that of rats maintained at 5 C with food ad libitum, respectively. These results indicate that the negative metabolic balance state of these cold conditions is not an active factor modifier of Δ6 desaturase activity, whereas it decreases Δ9 desaturase activity, reflecting the lipogenic characteristics of the latter enzyme.
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Different types of organisms from microorganisms to higher plants and poikilotherm animals respond to alterations in environmental temperature by modifying fatty acid composition and, thus, the physicochemical properties of their membranes. In some microorganisms, such as *Candida utilis* (1,2), *Bacillus megaterium* (3) and *Tetrahymena* (4), and in higher plants (5), polyunsaturated fatty acid synthesis is increased in response to a decrease in the environmental temperature.

It has also been demonstrated that the Δ6 desaturase activity of fish *(Pimelodus maculatus)* liver microsomes is greater in animals adapted to lower temperature (6). The effect of temperature on membrane fluidity is not as obvious in homeothermic animals as it is in poikilotherms.

Previous laboratory studies (7) in which female rats were adapted to warm temperature (30-32 C) for 20-25 days and then shifted to cooler temperature (13-15 C) for different periods of time showed that Δ9 desaturase activity decreased after 24 hr of cold exposure, while Δ6 desaturase activity was increased after this period of time. Male rats adapted to the same conditions did not show significant changes either in Δ6 or Δ9 desaturase.

Taking into account these results, it was thought that differences in Δ6 and Δ9 desaturase activity might appear in male rats by means of a change in the environment at temperatures lower than 12 C. In addition, we attempted to determine if the food intake and/or decreased weight gain of the animals kept in the cold environment might contribute to the changes in lipid desaturation and composition. For this purpose, male rats were transferred from 24 C to 5 C, and Δ6 and Δ9 desaturase activities and lipid composition of microsomes and mitochondria were determined.

MATERIALS AND METHODS

Materials. [1-^{14}C]Palmitic acid (53.8 mCi/mmol, 99% radiochemically pure), [1-^{14}C]stearic acid (56.0 mCi/mmol, 99% radiochemically pure) and [1-^{14}C]linoleic acid (54.7 mCi/mmol, 98.5% radiochemically pure, 1% *trans* isomer) were purchased from New England Nuclear (Boston, MA). Cofactors used for the enzymatic reactions were provided by Sigma Chemical Co. (St. Louis, MO), and all unlabeled fatty acids were from Nu-Chek-Prep Inc. (Elysian, MN). Silica Gel H (Kiesegel 60H) was purchased from Merck (Darmstadt, FRG). All chemicals and solvents were analytical grade.

Animal treatment. Male Wistar rats of 60-70 days of age weighing 180-200 g were maintained on a commercial standard pellet diet (Nutrimento rat chow 3, Escobar, Argentina) and tap water ad libitum and were housed in individual cages unless indicated otherwise. The fatty acid composition of the diet was as follows: 16.7% 16:0 (palmitic acid), 0.8% 16:1 (palmitoleic acid), 4.9% 18:0 (stearic acid), 21.8% 18:1 (oleic acid), 52.4% 18:2n-6 (linoleic acid) and 4.3% 18:3n-3 (linolenic acid).

When the influence of body weight changes and food intake on fatty acid metabolism was studied, experiments were carried out using four groups of four rats each monitoring daily weights and food intake. Group I comprised rats maintained at 22-24 C fed ad libitum (control ad libitum); group II was rats maintained in a cold temperature-controlled room at 5-6 C fed ad libitum (cold ad libitum); group III was rats maintained at 5-6 C whose food intake was restricted to match the food intake of group I (cold pair-fed to group I); and group IV was rats maintained at 24 C whose food intake was restricted so that their growth rate would match the growth rate of group II (control pair-weighed to group II). Food-restricted rats maintained at 5-6 C in group III were studied to determine whether changes in desaturation or lipid composition in rats maintained at the cold temperature might be due to increased food intake.

Since rats maintained in a cold environment have relative hyperphagia but gain less weight than those at 24 C, the food-restricted rats at 24 C (group IV) were studied to determine if changes in desaturation or lipid composition in rats maintained at cold temperatures might be due to their decreased growth rate and relatively increased fat mobilization. All animals were subjected to a daily photoperiod of 12 hr light and 12 hr darkness (midnight being the midpoint of the dark period). After five days under these conditions, animals were killed at 8 a.m. to equalize circadian effects (8), and liver microsomes and mitochondria were isolated.

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Fractionating procedures: preparation of microsomes. Microsomes were prepared from rats killed by decapitation. Livers were rapidly removed and homogenized in a cold solution (3:1, v/w) containing 0.25 M sucrose, 0.15 M KCl, 62 mM potassium phosphate buffer (pH 7.4), 5 mM $MgCl_2$ and 1.4 mM N-acetyl-L-cysteine. The homogenate was centrifuged at 10,000 × g for 20 min; the pellet was discarded and the resulting supernatant was centrifuged at 100,000 × g for 60 min to obtain the microsomal pellet. The microsomal fraction was resuspended in the same homogenizing solution to an appropriate protein concentration. In some instances, pellets were frozen and stored at −80 C for several weeks before use. All microsomal preparations were carried out at 0–4 C. When frozen in concentrated form (15 mg of microsomal protein/ml of homogenizing solution), enzyme activities were stable for up to a month. All subsequent assays and composition analyses used these microsomal membrane fractions.

Preparation of mitochondria. A portion of the liver was chilled immediately after removal by immersion in 0.25 M sucrose and 10 mM potassium phosphate buffer (pH 7.4) and was homogenized at 0–4 C (9:1, v/w). Liver mitochondria were prepared by differential centrifugation according to the method of Hogeboom (9).

Enzymatic assays. The Δ9 (16:0 → 16:1 and 18:0 → 18:1) and Δ6 (18:2n-6 → 18:3n-6) desaturase enzyme assays were carried out by measuring the conversion rate of the [1-^{14}C]labeled fatty acid substrate into the corresponding [1-^{14}C]labeled fatty acid product. Only the exogenous substrate added to the incubation mixture was considered for calculation of the enzymatic activity, since the amount of endogenous substrate (<7 nmol; only 1.5% [w/w] of microsomal lipids in all groups was free fatty acids) was negligible compared to the 50 nmol of exogenous substrate. Reactions were initiated by adding microsomal protein to preincubated flasks containing 0.25 M sucrose, 0.15 M KCl, 0.04 M potassium phosphate buffer (pH 7.2), 1.41 mM N-acetyl cysteine, 0.04 M KF, 1.3 mM ATP, 0.06 mM CoA, 0.87 mM NADH, 5 mM $MgCl_2$, 5 nmol of [1-^{14}C]labeled and 45 nmol of unlabeled acid in a final volume of 1.6 ml. For Δ9 desaturase assay, palmitic and stearic acids were used as substrates with 1.5 and 2 mg of microsomal protein. The substrate for Δ6 desaturase assays was linoleic acid and 3 mg of microsomal protein was used. The protein was determined by the Lowry procedure using crystalline bovine serum albumin as standard (10). The reaction mixture was incubated with constant shaking at 36 C for 15 min. The desaturation reaction was stopped by adding 2 ml of 10% KOH in ethanol. The fatty acids were recovered by saponification of the incubation mixture (45 min at 82 C), acidification and extraction with petroleum ether (bp 30–40 C). The fatty acids were esterified with methanolic 3 M HCl (1 hr at 64 C). The analyses were carried out by gas liquid radiochromatography in a Packard apparatus, Model 893, equipped with a proportional counter using 6P 10% SP-2330 on Chromosorb WAW (100–120 mesh) (11). A control assay without the addition of microsomes was done and no desaturation was observed.

Microsomal and mitochondrial lipid composition. Total microsomal and mitochondrial lipids were extracted using the method of Folch et al. (12), and methyl esters were prepared and analyzed by gas liquid chromatography (GLC) as previously described (13) in a Hewlett-Packard model 5840-A chromatograph, equipped with the 5840-A 6C terminal and using a 6-ft column filled with 10% SP 2330 on 100–200 mesh Chromosorb WAW. Peaks were identified by comparison of retention times with standards.

Phospholipids were separated by two-step thin layer chromatography (TLC) procedure using Silica Gel H plates (14). After the plate was exposed to iodine vapors, the phospholipid fractions were scraped off for quantification by colorimetric determination of phosphorus (15).

Statistical analysis. All results were tested for statistical significance by Student's t-test for paired comparison with control.

RESULTS

Food intake, growth rate, food conversion and liver weight in animals in a cold environment. Table 1 summarizes the daily food intake, growth rate and liver weight in the four groups. Although the rats kept at 5 C with food ad libitum (group II) showed an increase of 25% in food intake, they had a decrease in growth rate and food conversion of 74% and 86%, respectively, as a consequence of an increase in catabolism. In the cold environment, food calories are preferentially used maintaining body temperature.

Group III, animals maintained at 5 C with the same food intake as animals at 24 C, showed a decrease in the growth rate, food conversion and liver weight of 142%, 162% and 32%, respectively. The negative metabolic balance due to cold was even greater in this group as a consequence of the decrease in calories provided by the restricted food intake.

Rats of group IV, animals at 24 C and with food intake restricted to match the group-I growth rate, showed a decrease in food conversion and liver weight of 126% and 30%, respectively, since the restriction in food intake provoked an increase in the catabolism of endogenous substrates. The increased catabolism of groups II, III and IV must have been accompanied by increased lipolysis.

Influence of cold, temperature, food intake and growth rate on desaturation. The effect of cold and food restriction on the fatty acid desaturase activities is shown in Figure 1.

The enzymatic activity of Δ6 desaturase was not modified in the group at 5 C with food ad libitum, while it increased when food intake was restricted either at 5 C (group III) or at 24 C (group IV). Animals on the diet given ad libitum at 5 C (group II) showed a marked decrease not only in food conversion but also in the enzymatic activity of Δ9 desaturase.

It is hardly probable that the increase in food intake of rats kept at low temperatures should cause the changes observed in the enzymatic activity of Δ9 desaturase, since the restriction in food intake at 5 C (group III) emphasized rather than annulled the changes. On the other hand, the decrease in the growth rate of rats in the cold environment would be an important factor, since in rats kept at 24 C with a similar growth rate (group IV), a decrease in Δ9 desaturase enzymatic activity was also observed.

Groups showing a marked decrease in food conversion (II, III and IV) also had decreased Δ9 desaturase activity.

When rats were kept at 5 C for 15 days instead of five with food ad libitum, a behavior similar to that shown

TABLE 1

Effect of Temperature and Food Restriction on Food Intake, Growth Rate, Food Conversion and Liver Weight

	Group I, control ad libitum (n = 10)	Group II, cold ad libitum (n = 10)	Group III, cold pair-fed to group I (n = 4)	Group IV, control pair-weighed to group II (n = 4)
Starting weight (g)	197.0 ± 15.1	174.8 ± 14.4	192.5 ± 13.1	176.8 ± 16.0
Food intake (g/g)	0.108 ± 0.003	0.135 ± 0.004*	0.100 ± 0.004	0.064 ± 0.004*
Growth rate (g/day)	6.38 ± 0.36	1.68 ± 0.51*	−2.70 ± 0.30*	0.95 ± 0.70*
Food conversion	54.02 ± 5.03	7.48 ± 4.58*	−32.4 ± 4.4*	−13.7 ± 8.0*
Liver weight (g)	9.92 ± 0.80	8.44 ± 0.90	6.70 ± 0.35**	6.90 ± 0.42**

Food intake (daily food intake/body weight); growth rate (daily increase in body weight); food conversion (growth rate/food intake × 100) and liver weight. Animal treatments are described in Materials and Methods. Results are the mean ± S.E. n, represents the number of animals for each group. Significant differences from control: *, $p < 0.001$; **, $p < 0.01$.

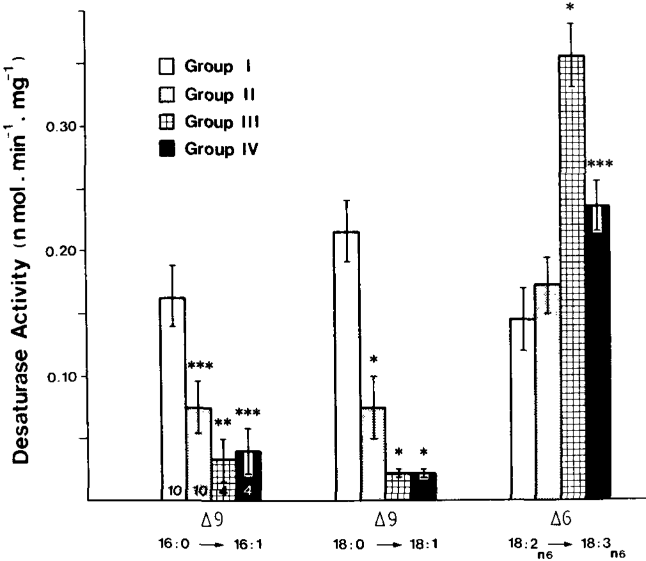

FIG. 1. Effect of environmental temperature and food restriction on hepatic microsomal fatty acid desaturase activity. Desaturase enzyme assays were carried out as described in Materials and Methods. Group I, control ad libitum; group II, cold ad libitum; group III, cold pair-fed to group I; group IV, control pair-weighed to group II. Results are shown as mean ± S.E. The number of animals in each group is shown in the first set of bars. Significantly different from controls: *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$.

in Figure 1 was observed in the enzymatic activity of Δ9 and Δ6 desaturases (results not shown).

Microsomal and mitochondrial lipid composition. Tables 2 and 3 show the lipid fatty acid composition of hepatic microsomes and mitochondria, respectively. The modifications observed in fatty acid composition of liver microsomes and mitochondria were generally similar.

The rats at 5 C with food ad libitum (group II) showed in both fractions increased percentage of 18:0 and decreased 18:1. Only in mitochondria did the percentage of 16:1 decrease significantly, and so did the unsaturation index in microsomes. These changes agree with the decrease found in microsomal Δ9 desaturase enzymatic activity in the same group.

In group III, the same modifications as in group II were observed in the percentages of 16:1, 18:0 and 18:1 in lipids of liver microsomes and mitochondria. As in group II, these changes are the consequences of a decrease in the enzymatic activity of Δ9 desaturase shown by group III. Only the microsomes had an increase in the percentage of 20:4n-6, which agreed with the one observed in the enzymatic activity of Δ6 desaturase (regulatory enzyme in the biosynthesis of polyunsaturated acids) (16).

Group IV presented an increased percentage of 18:0 and decreased 16:1 both in microsomes and mitochondria. The percentage of 18:1 decreased only in mitochondria. These changes are the consequences of the decrease observed in Δ9 desaturase enzymatic activity. Besides, both fractions showed an increase in the percentage of 20:4n-6 that could be attributed to an increase observed in the Δ6 desaturase enzymatic activity.

Table 4 shows the phospholipid composition of hepatic microsomes and mitochondria of rats kept at 24 C (group I) and 5 C (group II) on food ad libitum. No modification was observed in the phospholipid composition of liver microsomes, whereas in mitochondria the percentages of phosphatidylcholine (PC) and sphingomyelin (SM) decreased and that of phosphatidylethanolamine (PE) increased in rats kept in the cold environment. The changes found in liver mitochondria of rats kept at 5 C resembled those found in mitochondria of brown adipose tissue of rats kept at 4 C (17).

DISCUSSION

When a rat is exposed to a cold environment, adaptation processes are initiated characterized by shivering or nonshivering thermogenesis, depending on the conditions (18), tending to lower heat loss and to increase its production. There is an increase in thermogenesis (19), and the supply of substrates comes from an increase in fatty acid mobilization (by lipolysis of white and brown adipose tissue) (20–22) and from glucose (by liver glycogenolysis and gluconeogenesis) (23,24). In this substrate mobilization, the sympathetic nervous system takes part via catecholamines (18). Together with endogenous substrates, the exogenous ones coming from increased food intake also contribute to thermogenesis. This is the reason that when male rats are exposed to cold for five days, food intake increases and growth rate and food conversion decrease, since calories are preferentially used in heat production to keep body temperature.

TABLE 2

Effect of Environmental Temperature and Food Restriction on Liver Microsomal Fatty Acid Composition in Male Rats

Fatty acid (%)	Group I, control ad libitum	Group II, cold ad libitum	Group III, cold pair-fed to group I	Group IV, control pair-weighed to group II
16:0	21.4 ± 0.1	20.7 ± 0.4	20.2 ± 0.2**	18.9 ± 0.4*
16:1	2.1 ± 0.09	2.2 ± 0.20	1.5 ± 0.50	0.7 ± 0.40***
18:0	20.7 ± 0.2	23.5 ± 0.3*	23.8 ± 0.4*	24.3 ± 0.5*
18:1	10.2 ± 0.2	8.3 ± 0.3**	8.2 ± 0.4**	8.2 ± 1.7
18:2n-6	15.1 ± 0.4	15.3 ± 0.3	15.2 ± 0.5	14.7 ± 0.6
20:3n-6	2.5 ± 0.20	1.8 ± 0.20***	1.4 ± 0.09**	1.7 ± 0.20***
20:4n-6	21.0 ± 0.3	21.0 ± 0.6	22.8 ± 0.2**	25.5 ± 1.2**
22:5n-3	2.5 ± 0.20	2.1 ± 0.10	1.6 ± 0.08**	1.4 ± 0.20**
22:6n-3	4.6 ± 0.2	5.2 ± 0.3	5.3 ± 0.3	4.5 ± 0.4
Unsaturation index[a]	1.62 ± 0.01	1.58 ± 0.01***	1.62 ± 0.01	1.67 ± 0.03

Fatty acid composition was determined by gas liquid chromatography as described in Materials and Methods for the four groups of animals described in the text. Only major fatty acids were considered. Results are shown as the mean of 4 rats ± S.E.

[a]Unsaturation index = Σ number unsaturated mol × number double bonds/Σ number total mol. Significant differences from control: *, $p < 0.001$; **, $p < 0.01$, ***, $p < 0.5$.

TABLE 3

Effect of Environmental Temperature and Food Restriction on Liver Mitochondrial Fatty Acid Composition in Male Rats

Fatty acid (%)	Group I, control ad libitum	Group II, cold ad libitum	Group III, cold pair-fed to group I	Group IV, control pair-weighed to group II
16:0	20.0 ± 0.2	20.3 ± 0.5	17.8 ± 0.5**	17.3 ± 0.4*
16:1	2.0 ± 0.10	1.6 ± 0.10***	1.4 ± 0.08**	1.4 ± 0.06**
18:0	18.0 ± 0.3	20.7 ± 0.3*	21.3 ± 0.3*	21.1 ± 0.2*
18:1	10.9 ± 0.06	9.0 ± 0.30*	9.1 ± 0.20*	8.5 ± 0.20*
18:2n-6	21.7 ± 0.4	22.3 ± 0.2	22.0 ± 0.3	22.7 ± 0.7
20:3n-6	1.9 ± 0.10	1.3 ± 0.09**	1.5 ± 0.30	1.2 ± 0.08**
20:4n-6	19.7 ± 0.4	18.8 ± 0.3	20.8 ± 0.3	23.2 ± 0.9***
22:5n-3	2.1 ± 0.3	1.9 ± 0.2	1.9 ± 0.3	1.0 ± 0.1**
22:6n-3	3.7 ± 0.3	4.1 ± 0.2	4.2 ± 0.3	3.6 ± 0.3
Unsaturation index[a]	1.59 ± 0.01	1.54 ± 0.02	1.66 ± 0.03	1.70 ± 0.03

Fatty acid composition was determined by gas liquid chromatography as described in Materials and Methods for the four groups of animals described in the text. Only major fatty acids were considered. Results are shown as the mean of 4 rats ± S.E.

[a]Unsaturation index is explained in Table 2. Significant differences from control: *, $p < 0.001$; **, $p < 0.01$; ***, $p < 0.05$.

TABLE 4

Effect of Cold Environment on Phospholipid Composition of Microsomal and Mitochondrial Fractions % Phospholipid (μg/mg Total Lipid)

	Microsomes		Mitochondria	
	Group I, control ad libitum	Group II, cold ad libitum	Group I, control ad libitum	Group II, cold ad libitum
PC	59.3 ± 7.3	66.4 ± 3.1	52.1 ± 0.1	43.9 ± 0.5*
PE	21.2 ± 1.3	16.8 ± 0.9	22.5 ± 0.8	36.4 ± 1.1*
CL	—	—	15.2 ± 1.1	13.5 ± 0.8
PI	13.8 ± 4.0	11.6 ± 2.2	6.5 ± 0.5	5.4 ± 0.9
SM	3.1 ± 1.8	2.8 ± 1.1	1.8 ± 0.1	0.7 ± 0.2**
PS	2.6 ± 0.2	2.5 ± 1.3	1.9 ± 1.1	0.10 ± 0.02

PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidylinositol; SM, sphingomyelin; PS, phosphatidylserine. Phospholipids were separated as described in Materials and Methods. Results are expressed as the mean ± S.E. The number of samples analyzed is 3 for microsomal and 5 for mitochondrial fraction. Significant differences from control: *, $p < 0.001$; **, $p < 0.01$.

Δ6 Desaturase activity. Concerning the effect of environmental temperature on desaturating activity, no modification due to cold was observed either in Δ6 desaturase activity or in the percentage of linoleic acid in liver microsomal and mitochondrial lipids of male rats fed ad libitum. Hughes and York also observed no changes in the Δ6 desaturase enzymatic activity due to temperature in the lean mouse (25). These results also agree with those found by González et al. (7) when male rats were shifted from 30-32 C (adapted to that temperature for 20 days) to 12 C for five days, since in this case no modifications in the Δ6 desaturase were observed either. These results indicate that metabolic changes induced by an environmental temperature decrease expressed by a thermogenesis increase are not active factor modifiers of Δ6 desaturase activity. However, in female rats adapted to the same conditions, an increase in the enzymatic activity of Δ6 desaturase due to cold was observed and, in this change, estradiol levels would be involved (26). On the other hand, when the enzymatic activity in male rats was depressed by a hyperglycidic diet and animals were shifted from 24 C to 12 C, an increase was observed in Δ6 desaturase enzymatic activity due to cold environment (27).

The increase in Δ6 desaturase enzymatic activity observed in animals on a restricted diet, both at 5 C and at 24 C, was unexpected, and it was difficult to elucidate since fasting inhibits enzymatic activity (28,29). These results, as has been pointed out by Faas and Carter, emphasize the importance of food intake as another factor to be taken into account for the complex control of the desaturating system (30,31).

Δ9 Desaturase activity. González et al. found that Δ9 desaturase enzymatic activity was not modified when male rats were shifted to 13-15 C after being previously adapted to 30-32 C, whereas in female rats adapted to the same conditions, they observed a decrease in that desaturating activity (7). We have observed, however, that when male rats were kept at 5 C on food ad libitum (group II), the Δ9 desaturase activity decreased. This lowering was confirmed by a decrease in the percentages of 16:1 and 18:1 and an increase in 18:0 in the fatty acid composition of both liver microsomes and mitochondria. In rats kept at 5 C on a restricted diet (group III), the decrease in the Δ9 desaturase enzymatic activity was even more marked than at 5 C on food ad libitum. Moreover, a decrease in Δ9 desaturase activity was also detected in animals on a restricted food intake at 24 C (group IV).

In group II, there was a negative metabolic balance (decreased food conversion) and an increase in food intake to provide substrates for the thermogenesis process, which was increased by the cold temperature. In group III, the negative metabolic balance was even greater than in group II, since there was increased catabolism of endogenous substrates in the thermogenesis process due to the restriction imposed on food intake. Finally, group IV rats also had a negative metabolic balance to counterbalance food intake restriction enhancing the use of endogenous substrates.

In groups II, III and IV, characterized by a negative metabolic balance, the Δ9 desaturase enzymatic activity was diminished. It is known that Δ9 desaturase is very sensitive to nutritional (29,32-36) and hormonal (30,31,37,38) factors. Moreover, it is considered a lipogenic enzyme, since during fasting, when there is a negative metabolic balance and lipolysis is increased, its enzyme activity is decreased (29). In addition, lipogenic diets are the most efficient ones in increasing the abovementioned activity (29,36). Lipogenesis and Δ9 enzymatic activity respond in the same way to regulatory factors (39).

Therefore, it can be considered that the decrease observed in the Δ9 desaturase enzymatic activity in male rats at 5 C is the consequence of the negative metabolic balance state set off by these conditions.

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Formation of Diacyl and Alkylacyl Glycerophosphocholine in Rabbit Alveolar Macrophages

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The incorporation of various labeled precursors into alkenylacyl, alkylacyl and diacyl phospholipids in rabbit alveolar macrophages was studied. The incorporation rates of the individual precursors were shown to be quite different among the three subclasses of phospholipids. [^{3}H]Glycerol, [^{14}C]16:0, [^{14}C]18:1, [^{14}C]18:2 and [^{32}P]-orthophosphate were preferentially incorporated into choline glycerophospholipids (CGP), especially into diacyl glycerophosphocholine (GPC), indicating that the de novo synthesis of diacyl GPC is extremely high. Considerable portions of the radioactivities of [^{14}C]16:0, [^{14}C]18:1, [^{14}C]18:2 and [^{32}P]orthophosphate were also found in alkylacyl GPC, the incorporation being higher than or comparable to that in the case of diacyl glycerophosphoethanolamine (GPE). We then examined the activities of cholinephosphotransferase and ethanolaminephosphotransferase, and found that the activity of cholinephosphotransferase was remarkably high in macrophage microsomes compared with that in microsomes from several other tissues. This suggests that diradylglycerols were preferentially utilized by cholinephosphotransferase, which is consistent with the results obtained for intact cells. We confirmed that a considerably higher amount of diacyl GPC as well as alkylacyl GPC was formed through this enzyme reaction with macrophage microsomes than with brain microsomes. The high formation of alkylacyl GPC could be responsible, at least in part, for the accumulation of this unique ether phospholipid, a stored precursor form of platelet-activating factor in macrophages.
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Recently, it has been shown that considerable amounts of alkenyl and alkyl ether phospholipids are present in several types of inflammatory and immunological cells, such as macrophages, polymorphonuclear leukocytes, lymphocytes and platelets (reviewed in ref. 1). The occurrence of high amounts of alkylacyl GPC in these cells is particularly noteworthy, since the levels of alkylacyl GPC in other mammalian tissues are generally very low (1). The results of recent studies have revealed that this unique alkyl ether phospholipid is a stored precursor form of a potent lipid mediator, platelet-activating factor (PAF), and a degradation product of PAF in various types of cells (reviewed in ref. 2). Furthermore, alkenyl and alkyl ether phospholipids contain several times higher amounts of C_{20} and C_{22} polyunsaturated fatty acids than diacyl species in certain cell types (1). Ether phospholipids could be important pools of these polyunsaturated fatty acids in cells. In fact, large amounts of 20:4 were released from both diacyl and ether phospholipids in various types of cells upon stimulation (3–7).

Although the significance of alkylacyl GPC in inflammatory and immunological cells has recently received much attention, little information is yet available on the metabolism of alkylacyl GPC in these types of cells. In particular, very little is known about the de novo synthesis of this unique alkyl ether lipid, abundant in white blood cells and platelets. Moreover, previous studies on the metabolism of alkenylacyl, alkylacyl and diacyl phospholipids were mainly performed on the phospholipids in nervous tissues and tumor cells, and there has so far been no precise comparative study on the metabolism of diacyl and ether phospholipids in inflammatory and immunological cells.

In this study, we investigated the incorporation of various labeled precursors for the glycerol backbone, fatty acyl residues and polar head groups into alkenylacyl, alkylacyl and diacyl phospholipids in rabbit alveolar macrophages. The activities of the de novo synthetic enzymes, cholinephosphotransferase and ethanolaminephosphotransferase, were also examined using endogenous or exogenous diradylglycerols. The active formation of diacyl and ether-containing CGP was discussed.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade, and solvents were distilled before use. The precoated silica gel plates were purchased from Merck (Darmstadt, FRG). [1(3)-^{3}H]Glycerol (3 Ci/mmol), [1-^{14}C]palmitic acid (58 mCi/mmol), [1-^{14}C]stearic acid (60 mCi/mmol), [1-^{14}C]oleic acid (57 mCi/mmol), [1-^{14}C]linoleic acid (57 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (100 Ci/mmol), [methyl-^{14}C]choline chloride (50.5 mCi/mmol) and [1-^{3}H]ethanolamine hydrochloride (23 Ci/mmol) were purchased from Amersham (Amersham, UK). [U-^{14}C]Docosahexaenoic acid (160 mCi/mmol), CDP-[methyl-^{14}C]choline (42.35 mCi/mmol) and CDP-[2-^{14}C]ethanolamine (27 mCi/mmol) were from New England Nuclear (Boston, MA). ^{32}P-Orthophosphate (carrier-free) was from the Japan Isotope Center (Tokyo, Japan). Selachylalcohol (1-monooleyl glycerylether) was purchased from Serdary Research Lab. (Ontario, Canada). Docosahexaenoic acid (22:6) was from Nu-Chek-Prep (Elysian, MN). HEPES was obtained from Wako Pure Chemical Industry (Tokyo, Japan). Eagle's minimal essential medium (MEM) was obtained from Nissui Seiyaku Co. (Tokyo, Japan). Oleic acid (18:1), arachidonic acid (20:4), oleic anhydride, 1,2-dioleoyl-*sn*-glycerol, essentially fatty acid–free bovine serum albumin (BSA), CDP-choline and CDP-ethanolamine were obtained from Sigma (St. Louis, MO). 1-Alkyl(18:1)-2-oleoyl-*sn*-glycerol was prepared as follows: selachylalcohol was acylated with oleic anhydride in pyridine/chloroform (1:5, v/v) at room temperature for 12 hr. The

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Abbreviations: CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; IGP, inositol glycerophospholipids; SGP, serine glycerophospholipids; Sph, sphingomyelin; lyso-bis-PA, lyso-bis-phosphatidic acid; CL, cardiolipin; GPC, glycero-3-phosphocholine; GPE, glycero-3-phosphoethanolamine; PAF, platelet-activating factor; BSA, bovine serum albumin; TLC, thin layer chromatography; GLC, gas liquid chromatography. Fatty chains are designated in terms of number of carbon atoms:number of double bonds, e.g., 18:1 for oleic acid.

resultant 1-alkyl-2,3-diacylglycerol was purified by thin layer chromatography (TLC) with development with petroleum ether/ethyl ether/acetic acid (90:10:1, v/v/v), and then hydrolyzed with Rhizopus delemar lipase (Seikagaku Kougyo Co., Tokyo, Japan) to remove the fatty acid at the 3-position, as described previously (8). The 1-alkyl-2-acyl compound was purified by TLC with development with petroleum ether/ethyl ether/acetic acid (60:40:1, v/v/v), and then extracted from the silica gel with chloroform/methanol (1:1, v/v). The quantities of diradylglycerols were determined from the fatty acyl moieties as fatty acid methyl esters by gas liquid chromatography (GLC) (8,9).

Cells. Macrophages were prepared from healthy female rabbits (New Zealand white, 2.5–3 kg) as described previously (9). The macrophages were washed three times with saline after the removal of contaminating erythrocytes by means of osmotic lysis. The purity of the cells was above 95%, as judged on microscopic examination.

Incorporation of labeled precursors. Macrophages were suspended in 20 mM HEPES-MEM medium (pH 7.2) at 10^6 cells/ml. [^{3}H]20:4, [^{14}C]22:6 and [^{3}H]ethanolamine were diluted with the respective nonradiolabeled fatty acid at 50 mCi/mmol prior to use. The labeled fatty acids (1 μCi) were dissolved in 250 μl of 0.2% BSA-containing saline, and [^{3}H]glycerol (5 μCi), [^{32}P]orthophosphate (100 μCi), [^{14}C]choline (1 μCi) and [^{3}H]ethanolamine (1 μCi) were dissolved in 250 μl of saline. Each labeled compound (250 μl) was added to 5 ml of cell suspension. The incubation was carried out at 37 C for 60 min and terminated by adding 20 ml of chloroform/methanol (1:2, v/v).

Lipid analyses. Total lipids were extracted by the method of Bligh and Dyer (10). Individual phospholipids were separated by two-dimensional TLC with development with chloroform/methanol/28% NH_4OH (65:35:5, v/v/v) for the first dimension and chloroform/acetone/methanol/acetic acid/water (5:2:1:1.3:0.5, v/v/v/v/v) for the second dimension. Neutral lipids were separated by TLC with development with petroleum ether/ethyl ether/acetic acid (80:20:1, v/v/v). The alkenylacyl, alkylacyl and diacyl lipid classes were separated as 1,2-diradyl-3-acetylglycerol derivatives as described elsewhere (7,8). In brief, CGP and EGP were first hydrolyzed with phospholipase C *(Bacillus cereus)* to remove the polar groups. The resultant diradylglycerols were further acetylated with acetic anhydride and pyridine. The 1,2-diradyl-3-acetylglycerols were separated into the alkenylacyl, alkylacyl and diacyl classes by TLC with development with petroleum ether/ethyl ether/acetic acid (90:10:1, v/v/v) and then with toluene. The lipid spots were visualized under ultraviolet light after spraying with 0.001% primuline in acetone/water (4:1, v/v) and then were scraped off from the silica gel plate into counting vials. The radioactivity was estimated with a scintillation counter (Aloka, Model LSC 903) using a scintillation fluid, as described earlier (11). In some experiments, the successive mild alkaline and acid hydrolysis method was used to determine the radioactivities of alkenylacyl, alkylacyl and diacyl compounds. Lipid phosphorus was estimated by the method of Rouser et al. (12).

Preparation of microsomal fraction. Microsomal fraction was prepared from various tissues and cells. Rabbit alveolar macrophages were prepared from rabbits that had been injected with 1.0 ml of Freund's complete adjuvant 3 wk prior to sacrifice. Pig lymphocytes were prepared from pig mesenteric lymph nodes as described previously (8). Both types of cells were washed three times with saline. Various tissues and cells were homogenized in a Potter-Elvejhem homogenizer with a Teflon pestle in 0.25 M sucrose–0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA. The homogenate was centrifuged at 10,000 × g for 20 min twice. The supernatant was collected and ultracentrifuged at 105,000 × g for 60 min. The pellet was resuspended in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM EDTA and then washed once at 105,000 × g for 60 min. The pellet (microsomal fraction) was resuspended in the same buffer and stored at −20 C. The protein content was estimated by the method of Lowry et al. (13).

Enzyme assays. The activities of cholinephosphotransferase and ethanolaminephosphotransferase were determined by the modified method of Lee et al. (14). The incubation mixture contained CDP-[methyl-^{14}C]choline or CDP-[2-^{14}C]ethanolamine (0.5 mM, 0.025 μCi), $MgCl_2$ (8 mM), BSA (0.5 mg), EGTA (0.5 mM) and microsomes (0.5 mg protein) in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0), with endogenous diradylglycerols as the substrates. When exogenous diradylglycerols were used as the substrates, the incubation mixture consisted of CDP-[methyl-^{14}C]choline or CDP-[2-^{14}C]ethanolamine (0.02 mM–0.2 mM), $MgCl_2$ (8 mM), BSA (0.5 mg), EGTA (0.5 mM), 1-alkyl-2-acylglycerol or 1,2-diacylglycerol (4 mM) and microsomes (0.1 mg protein) in 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.0). The alkylacylglycerol or diacylglycerol was sonicated in 0.04% Tween 20 in water with a Branson sonifier (four periods of 10 sec each) immediately before use. The incubation was performed at 37 C for 30 min and terminated by adding chloroform/methanol (1:2, v/v). The lipids were extracted and then separated by TLC with development with chloroform/methanol/water (65:25:4, v/v/v). The radioactivities in the CGP and EGP fractions were estimated as described above. Portions of CGP and EGP were further fractionated by means of the successive mild alkaline and acid hydrolysis method (15) to determine the radioactivities of alkenylacyl, alkylacyl and diacyl compounds.

RESULTS AND DISCUSSION

Incorporation of various labeled precursors into individual phospholipids. Table 1 shows the incorporation rates into the cellular phospholipid fraction of various fatty acids. [^{3}H]20:4 and [^{14}C]22:6 were incorporated very rapidly into the cellular lipid fractions during the incubation. On the other hand, the incorporation rates of [^{14}C]16:0, [^{14}C]18:0, [^{14}C]18:1 and [^{14}C]18:2 were relatively slow.

Table 2 shows the distribution of the radioactivities of labeled precursors among individual phospholipids. [^{3}H]Glycerol, [^{32}P]orthophosphate, [^{14}C]16:0, [^{14}C]18:1 and [^{14}C]18:2 were preferentially incorporated into CGP. Radioactivities other than those in CGP were low, except for that of [^{14}C]18:2 in lyso-bis-phosphatidic acid (lyso-bis-PA). Large portions of the radioactivities of [^{3}H]20:4, [^{14}C]22:6 and [^{14}C]18:0 were also distributed in CGP, although considerable radioactivities were found as well in EGP ([^{3}H]20:4, [^{14}C]22:6 and [^{14}C]18:0), lyso-bis-PA ([^{3}H]20:4 and [^{14}C]22:6) and IGP ([^{3}H]20:4 and [^{14}C]18:0). These observations indicate that the formation of CGP,

TABLE 1

Incorporation Rates of Various Labeled Fatty Acids into Cellular Phospholipid Fraction[a]

Fatty acids	%	nmol
[^{14}C]16:0	7.5 ± 0.7	0.26
[^{14}C]18:0	8.9 ± 0.4	0.30
[^{14}C]18:1	7.6 ± 0.3	0.27
[^{14}C]18:2	15.9 ± 0.5	0.56
[^{3}H]20:4	54.9 ± 0.5	2.20
[^{14}C]22:6	79.9 ± 1.2	3.20

[a]Macrophages (10^6 cells/ml) were incubated with various labeled fatty acids (0.2 μCi/ml) at 37 C for 60 min as described in Materials and Methods. The values represented the incorporation rates (% and nmol/10^6 cells) of exogenously added labeled fatty acids into cellular phospholipid fraction. The mean percentages ± SD were calculated for three determinations.

particularly that via the de novo pathway, is very active compared with that of other phospholipids in this type of cell. The relatively high incorporation of [^{3}H]20:4 and [^{14}C]18:0 into EGP and IGP could be closely related to the fact that these phospholipids contain high amounts of 18:0, especially at the 1-position, and 20:4, at the 2-position. In contrast, the level of the 18:0 acid in CGP has been shown to be very low in this type of cell (9). Albert and Snyder (5) have already shown the preferential labeling of CGP with [^{14}C]16:0, [^{14}C]18:1, [^{14}C]18:2 and [^{14}C]20:4 in comparison with other phospholipids in rat alveolar macrophages.

Incorporation of various labeled precursors into subclasses of CGP and EGP. No precise data are available on the incorporation of various labeled precursors, except for 20:4 and 22:6 into alkenylacyl, alkylacyl and diacyl phospholipids in inflammatory and immunological cells. Table 2 shows the distribution of the radioactivities of various precursors among the alkenylacyl, alkylacyl and diacyl classes of CGP and EGP. The proportions of alkenylacyl, alkylacyl and diacyl compounds were 5.6%, 32.5% and 61.9% for CGP, and 61.2%, 8.1% and 30.7% for EGP, respectively, as reported previously (9). It should be noted that the incorporation rates of individual precursors were quite different between the subclasses of CGP and EGP. Most of the radioactivities of [^{3}H]-glycerol, [^{14}C]16:0, [^{14}C]18:0, [^{14}C]18:1, [^{14}C]18:2, [^{32}P]-orthophosphate and [^{14}C] choline were found in diacyl species in CGP, indicating that the de novo synthesis of diacyl GPC is extremely high for this type of cell. The radioactivity in diacyl GPE was also highest among the subclasses of EGP labeled with [^{3}H]glycerol, [^{14}C]16:0, [^{14}C]18:0, [^{14}C]18:1 and [^{14}C]18:2. Interestingly, significant portions of the radioactivities of various precursors were found in alkylacyl GPC, which is a stored precursor form of PAF. For instance, 6.1% ([^{14}C]16:0), 2.6% ([^{14}C]18:1), 1.6% ([^{14}C]18:2) and 2.5% ([^{32}P]orthophosphate) of the respective radioactivities in total phospholipids were distributed in alkylacyl GPC (Tables 2 and 3). The incorporation rate of [^{14}C]18:1 into alkylacyl GPC was calculated to be almost comparable to that into diacyl GPE (2.2% of the radioactivities in total phospholipids). These observations suggest that significant amounts of alkylacyl GPC are formed via the de novo pathway in this type of cell. In the case of [^{3}H]glycerol, however, it is necessary to take into account the fact that ether phospholipids are only synthesized via the dihydroxyacetone phosphate pathway (2). This may explain, at least in part, the generally low incorporation of [^{3}H]glycerol into ether-containing compounds. Furthermore, the low specific activity of alkenylacyl GPE labeled with [^{3}H]glycerol could be due to the fact that alkenylacyl GPE is formed from alkylacyl GPE through desaturation of the alkyl chain.

It is noteworthy that the distributions of the radioactivities of [^{32}P]orthophosphate and [^{14}C]ethanolamine in ether-containing EGP were somewhat higher than those of [^{3}H]glycerol, [^{14}C]16:0, [^{14}C]18:0, [^{14}C]18:1 and [^{14}C]18:2 (Table 3). These observations suggest that the polar head group of ether-containing EGP is turned over rapidly compared with that of diacyl species. The exchange of the phosphoryl base moiety rather than the base group could solely take place, since high labeling was observed with both [^{32}P]orthophosphate and [^{3}H]ethanolamine. This is also the case for alkenylacyl GPC labeled with [^{32}P]-orthophosphate and [^{14}C]choline. The backward action of

TABLE 2

Distribution of the Radioactivities of Various Labeled Precursors among Phospholipids in Rabbit Alveolar Macrophages[a]

Precursors	CGP (%)	EGP (%)	IGP (%)	SGP (%)	Sph (%)	Lyso-bis-PA (%)	CL (%)	PA (%)
[^{3}H]Glycerol	93.2 ± 1.6	3.4 ± 1.0	1.1 ± 0.2	0.2 ± 0.1	0.5 ± 0.3	1.2 ± 0.4	0.2 ± 0.2	0.2 ± 0.0
[^{14}C]16:0	93.1 ± 1.6	2.8 ± 0.4	1.7 ± 0.6	0.3 ± 0.1	0.9 ± 0.3	1.0 ± 0.6	0.0 ± 0.0	0.2 ± 0.1
[^{14}C]18:0	47.5 ± 1.1	18.1 ± 0.2	27.9 ± 1.0	5.0 ± 0.1	0.3 ± 0.0	0.7 ± 0.0	0.4 ± 0.1	0.1 ± 0.0
[^{14}C]18:1	85.9 ± 2.4	2.8 ± 0.8	3.2 ± 0.3	1.1 ± 0.3	0.5 ± 0.1	6.2 ± 1.5	0.1 ± 0.0	0.2 ± 0.0
[^{14}C]18:2	71.4 ± 1.8	2.8 ± 0.4	0.9 ± 0.4	0.6 ± 0.1	0.1 ± 0.0	23.8 ± 1.2	0.2 ± 0.0	0.2 ± 0.1
[^{3}H]20:4	58.4 ± 3.3	18.6 ± 3.5	11.5 ± 1.6	0.8 ± 0.0	0.9 ± 0.7	9.6 ± 1.6	0.1 ± 0.0	0.1 ± 0.0
[^{14}C]22:6	44.0 ± 1.8	35.8 ± 3.0	0.4 ± 0.0	0.6 ± 0.2	0.3 ± 0.0	18.7 ± 1.5	0.1 ± 0.0	0.1 ± 0.0
[^{32}P]Orthophosphate	90.7 ± 2.2	0.8 ± 0.0	1.1 ± 0.0	0.2 ± 0.1	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	6.8 ± 2.0

CGP, EGP, IGP and SGP: choline, ethanolamine, inositol and serine glycerophospholipids, respectively; Sph, sphingomyelin; lyso-bis-PA, lyso-bis-phosphatidic acid; CL, cardiolipin; PA, phosphatidic acid.

[a]Macrophages (10^6 cells/ml) were incubated with various labeled precursors (0.2 μCi/ml for fatty acids, 0.2 μCi/ml for [^{3}H]glycerol and 20 μCi/ml for [^{32}P]orthophosphate) at 37 C for 60 min. The incubation was terminated by adding chloroform/methanol (1:2, v/v), and then the total lipids were extracted by the method of Bligh and Dyer (10). Individual phospholipids were separated by two-dimensional thin layer chromatography as described in Materials and Methods. The mean percentages ± SD were calculated for three determinations.

TABLE 3

Distribution of the Radioactivities of Various Labeled Precursors in Subclasses of Choline (CGP) and Ethanolamine (EGP) Glycerophospholipids[a]

	CGP (%)			EGP (%)		
Precursors	Alkenylacyl	Alkylacyl	Diacyl	Alkenylacyl	Alkylacyl	Diacyl
[^{3}H]Glycerol	0.04 ± 0.01 (0.007)	0.06 ± 0.01 (0.002)	99.90 ± 0.02 (1.61)	2.0 ± 0.5 (0.03)	1.6 ± 0.2 (0.20)	96.4 ± 0.7 (3.14)
[^{14}C]16:0	0.3 ± 0.1 (0.05)	6.6 ± 0.8 (0.20)	93.1 ± 0.8 (1.50)	11.5 ± 2.5 (0.19)	11.0 ± 1.1 (1.36)	77.5 ± 3.0 (2.52)
[^{14}C]18:0	0.2 ± 0.0 (0.04)	1.4 ± 0.0 (0.04)	98.4 ± 0.1 (1.59)	0.4 ± 0.0 (0.01)	0.2 ± 0.1 (0.02)	99.4 ± 0.1 (3.24)
[^{14}C]18:1	0.2 ± 0.1 (0.04)	3.0 ± 0.1 (0.09)	96.8 ± 0.1 (1.56)	18.3 ± 1.4 (0.30)	1.6 ± 0.2 (0.20)	80.1 ± 1.6 (2.61)
[^{14}C]18:2	0.3 ± 0.1 (0.05)	2.3 ± 0.1 (0.07)	97.4 ± 0.2 (1.57)	6.1 ± 0.6 (0.10)	1.9 ± 0.5 (0.23)	92.0 ± 0.1 (3.00)
[^{3}H]20:4	1.8 ± 0.2 (0.32)	47.0 ± 1.2 (1.45)	51.2 ± 1.2 (0.83)	56.4 ± 1.3 (0.92)	6.3 ± 1.6 (0.78)	37.3 ± 1.3 (1.21)
[^{14}C]22:6	1.5 ± 0.2 (0.27)	33.8 ± 1.3 (1.04)	64.7 ± 1.4 (1.05)	44.5 ± 1.4 (0.73)	8.8 ± 0.6 (1.09)	46.7 ± 0.6 (1.52)
[^{32}P]Orthophosphate	1.5 ± 0.2 (0.27)	2.8 ± 1.0 (0.09)	95.7 ± 1.0 (1.55)	41.6 ± 5.2 (0.68)	22.2 ± 4.5 (2.74)	36.2 ± 5.2 (1.18)
[^{14}C]Choline or [^{3}H]ethanolamine[b]	5.7 ± 1.7 (1.02)	9.1 ± 3.0 (0.28)	85.2 ± 3.4 (1.38)	21.6 ± 0.6 (0.35)	12.3 ± 2.6 (1.52)	66.1 ± 2.2 (2.15)

[a]Macrophages (10^6 cells/ml) were incubated with various labeled precursors at 37 C for 60 min. The alkenylacyl, alkylacyl and diacyl subclasses of CGP and EGP were separated as described in Materials and Methods. The mean percentages ± SD were calculated for three determinations. The values in parentheses are the relative specific activities (cpm %/wt %) of three subclasses of CGP and EGP, respectively.
[b][^{14}C]Choline for CGP and [^{3}H]ethanolamine for EGP.

phosphotransferases or phospholipase C could be involved in the turnover of polar head groups in ether-containing compounds. The relatively high turnover of phosphate in ether-containing phospholipids has also been reported for Ehrlich ascites tumor cells (16), Krebs II ascites cells (17) and rabbit neuronal or glial cells (18). However, the biological significance and the precise mechanism of these findings still remain to be clarified.

Contrary to the case for other labeled fatty acids, considerable labeling of alkylacyl GPC and alkenylacyl GPE was observed with [^{3}H]20:4 and [^{14}C]22:6 (Table 3). This suggests that the incorporation mechanism for [^{3}H]20:4 and [^{14}C]22:6 is quite different from those in the case of other labeled fatty acids. In fact, the percent distributions of the radioactivities of [^{14}C]18:2 among alkenylacyl, alkylacyl and diacyl GPC and GPE did not seriously change up to 120 min (data not shown), whereas that of [^{3}H]20:4 changed markedly during the incubation (Fig. 1). The radioactivity in diacyl GPC decreased after 15 min, although 25% of the radioactivity remained in the form of free [^{3}H]20:4. The radioactivities in alkenylacyl GPE and alkylacyl GPC continued to gradually increase. Similar incorporation profiles were observed for 22:6 in rabbit alveolar macrophages (data not shown).

The alteration in the distribution of the radioactivities among diacyl and ether phospholipids could be due to the transacylation of polyunsaturated fatty acids between phospholipids (19-30). It is likely that large portions of 20:4 and 22:6 in ether phospholipids are incorporated via the transacylation pathway in addition to the Lands pathway. We have already shown that macrophage microsomes contain a high transacylase activity, which was almost comparable to the activity of acyl-CoA:1-alkyl-GPC acyltransferase (30). The most effective acyl donor in the transacylation system was diacyl GPC (30) and so the very active formation of diacyl GPC (Tables 2 and 3 and Fig. 1) may be coupled with the transfer of polyunsaturated fatty acids to ether lysophospholipids through the transacylation reaction. However, the possibility cannot be ruled out that portions of polyunsaturated fatty acids, especially 22:6, were esterified to ether phospholipids through the de novo synthetic pathway, since both 20:4-CoA and 22:6-CoA were effective substrates for the acylation of 1-alkyl-*sn*-glycero-3-phosphate (31). In addition, it is evident that 22:6-containing diradylglycerols were preferentially utilized in the formation of EGP by ethanolaminephosphotransferase (32,33). These 22:6-containing diradylglycerols could be formed via the de novo pathway. In fact, very rapid and transient labeling of diradylglycerols with [^{14}C]22:6 has been observed in this cell (27).

Formation of CGP and EGP by choline- and ethanolaminephosphotransferases. As demonstrated in Tables 2 and 3, various precursors were very rapidly incorporated into CGP, particularly the diacyl subclass, in rabbit alveolar macrophages. This observation prompted us to further investigate the enzyme activity involved in the de novo synthesis of CGP, and to compare it to that in

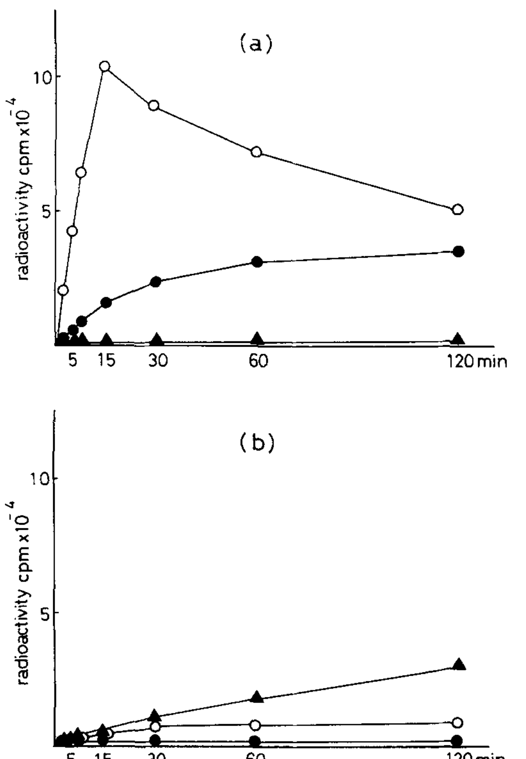

FIG. 1. The incorporation of [^{3}H]20:4 into the alkenylacyl, alkylacyl and diacyl subclasses of choline glycerophospholipid (a) and ethanolamine glycerophospholipid (b). Macrophages (10^6 cells/ml) were incubated with [^{3}H]20:4 (0.2 μCi/ml) at 37 C. Lipids were extracted and then fractionated as described and in Materials and Methods. The data are representative of three separate experiments. Alkenylacyl (▲), alkylacyl (●) and diacyl (○) subclasses.

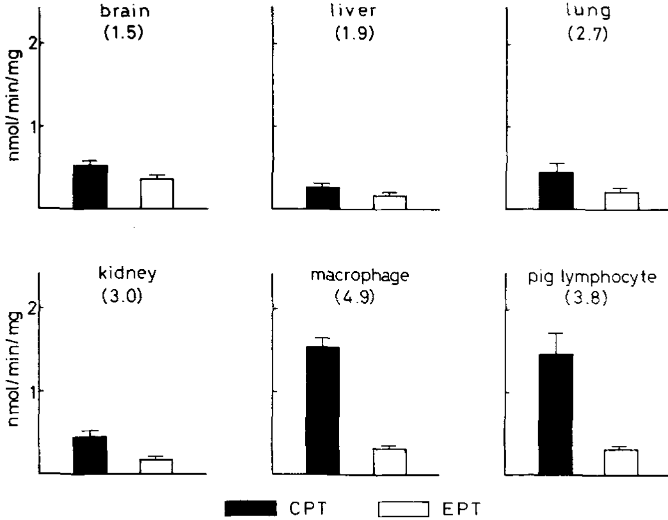

FIG. 2. The formation of choline and ethanolamine glycerophospholipids by cholinephosphotransferase (CPT) and ethanolamine-phosphotransferase (EPT) in microsomes prepared from various rabbit tissues and pig lymphocytes. Endogenous microsomal diradylglycerols were used as substrates as described in Materials and Methods. The mean values ± SD were calculated for three determinations. The values in parentheses are the ratios of the activity of CPT to that of EPT.

other tissues. First, we examined the activities of cholinephosphotransferase and ethanolaminephosphotransferase, which catalyze the final step of the de novo synthesis of CGP and EGP, respectively, with endogenous microsomal diradylglycerols as substrates. The formation of [^{14}C]labeled CGP and EGP from CDP-[^{14}C]choline and CDP-[^{14}C]ethanolamine was proportional to the amounts of protein added to 5 mg (in the absence of exogenous diradylglycerols) and 0.1 mg (in the presence of diradylglycerols) (data not shown). The reaction was also linear with time up to 30 min when 0.5 mg (in the absence of exogenous diradylglycerols) and 0.1 mg (in the presence of diradylglycerols) of microsomal protein was employed. Hence, the microsomal protein and the incubation time were limited to 0.5 mg (without exogenous diradylglycerol) and 0.1 mg (with exogenous diradylglycerol) and 30 min, respectively, in the following experiments.

Figure 2 shows the activities of the choline- and ethanolaminephosphotransferases in microsomal fraction from several rabbit tissues and cells and pig lymphocytes with endogenous diradylglycerols as substrates. Comparable activities of cholinephosphotransferase were observed for microsomal fractions from the brain, lung and kidney. The activity in liver was somewhat lower than those in these three tissues. On the other hand, macrophage microsomal fraction exhibited remarkably higher cholinephosphotransferase activity. The formation of labeled CGP by macrophage microsomes was ca. 3–6 times higher than that by microsomes from brain, liver, lung and kidney. Similar high activity was detected for pig lymphocyte microsomal fraction. As for ethanolaminephosphotransferase, the differences in the activities were not so pronounced between macrophage microsomal fraction and those from other tissues as in the case of cholinephosphotransferase. The activities were rather similar for the liver, kidney and lung microsomal fractions. The macrophage microsomal fraction and the brain and pig lymphocyte microsomal fractions exhibit ethanolaminephosphotransferase activity only ca. two times higher compared with these three tissues.

Although the levels of diradylglycerols available in the microsomal fraction may affect the rates of formation of CGP and EGP, the limited quantities of membrane lipids did not allow for accurate measurement of the amounts of endogenous diradylglycerols. Nevertheless, we confirmed that macrophage microsomal fraction exhibits considerably higher cholinephosphotransferase activity than brain microsomal fraction with 4 mM exogenous diacylglycerol or alkylacylglycerol. The Vmax of cholinephosphotransferase were 16.6 nmol/min/mg (macrophages) and 3.3 nmol/min/mg (brain) with diacylglycerol and 14.3 nmol/min/mg (macrophages) and 4.0 nmol/min/mg (brain) with alkylacylglycerol, respectively. The apparent Km values for CDP-choline were 41.2 μM (macrophages) and 36.6 μM (brain) with diacylglycerol and 51.3 μM (macrophages) and 40.0 μM (brain) with alkylacylglycerol, respectively.

These observations suggest that endogenous diradylglycerols were preferentially utilized by cholinephosphotransferase rather than ethanolaminephosphotransferase in rabbit alveolar macrophages. This is in good agreement with the results shown in Table 2 showing that various labeled precursors were preferentially

TABLE 4

Formation of the Alkenylacyl, Alkylacyl and Diacyl Subclasses of CGP and EGP by Phosphotransferases[a]

	Macrophages (pmol/min/mg)		Brain (pmol/min/mg)	
	CPT	EPT	CPT	EPT
Alkenylacyl	121 ± 28	60 ± 6	116 ± 20	109 ± 11
Alkylacyl	195 ± 33	92 ± 16	27 ± 7	32 ± 5
Diacyl	1253 ± 181	168 ± 33	315 ± 55	238 ± 30

CGP, choline glycerophospholipid; EGP, ethanolamine glycerophospholipid; CPT, cholinephosphotransferase; EPT, ethanolaminephosphotransferase.

[a]The formation of the subclasses of CGP and EGP by microsomes was examined with endogenous diradylglycerols as substrates. The alkenylacyl, alkylacyl and diacyl phospholipids were separated as described in Materials and Methods. The mean values ± SD were calculated for three determinations.

incorporated into CGP. Pig lymphocytes also exhibit higher cholinephosphotransferase activity and the preferential labeling of CGP with various labeled precursors (Sugiura, T., unpublished results). Furthermore, certain types of white blood cells have been shown to synthesize CGP actively (34-38). It seems possible that the high turnover of CGP in these cells is related to some biological function.

Formation of alkenylacyl, alkylacyl and diacyl subclasses of CGP and EGP by phosphotransferases. Then we separated CGP and EGP, newly formed through the action of the phosphotransferases with endogenous diradylglycerols, into the alkenylacyl, alkylacyl and diacyl subclasses. Table 4 shows the formation of the three subclasses of CGP and EGP by macrophage and brain microsomal fraction. Marked formation of diacyl GPC was observed with macrophage microsomes. Although a considerable amount of alkylacyl GPC was synthesized by macrophage microsomes, the formation was lower with brain microsomes. The ratio of the formation of alkylacyl GPC with macrophage microsomal fraction versus that with brain microsomal fraction was 7.2. On the other hand, the ratios of the synthesis of diacyl, alkylacyl and alkenylacyl GPE with macrophage microsomal fraction to those with brain microsomal fraction were 0.7, 2.9 and 0.6, respectively.

The activities of cholinephosphotransferase and ethanolaminephosphotransferase toward endogenous and exogenous diacylglycerol and alkylacylglycerol have been studied by several investigators (14,39-47). Freysz et al. (47) demonstrated the relatively higher affinity of ethanolaminephosphotransferase for CDP-ethanolamine than that of cholinephosphotransferase for CDP-choline, in the presence of alkylacylglycerol in chicken brain. They suggested that alkylacylglycerol was preferentially utilized by ethanolaminephosphotransferase to yield alkylacyl GPE, followed by desaturation to form alkenylacyl GPE, differing from the case of diacylglycerol. In fact, it is well known that brain tissues contain high amounts of ether-containing compounds only in EGP (1). We showed here that endogenous alkylacylglycerol was effectively utilized by both cholinephosphotransferase and ethanolaminephosphotransferase in macrophage microsomal fraction. The relatively high synthetic rates of alkylacyl GPC could be closely related to the remarkably high cholinephosphotransferase activity in macrophages. It has already been assumed that the same enzyme catalyzes the transfer of CDP-choline to either alkylacylglycerol or diacylglycerol (14,39-43,45,47). Furthermore, it seems possible that the active formation of alkylacyl GPC (Tables 2-4) could be responsible, at least in part, for the high abundance of this phospholipid in macrophages, although the degradation of alkylacyl GPC in these cells has not yet been examined. Pig lymphocytes also contain high amounts of alkylacyl GPC (8) and exhibit high cholinephosphotransferase activity (Fig. 2). Alkylacylglycerol could be utilized not only for the synthesis of ether-containing EGP but also for that of alkylacyl GPC in these types of cells.

In any case, the high synthesis and accumulation of alkylacyl GPC in macrophages is of particular importance, since alkylacyl GPC is regarded as the stored precursor form of PAF, which is now considered to be an important lipid mediator in inflammation and hypersensitivity. Several investigators have pointed out the possible involvement of PAF in the pathogenesis of asthma (48-51). Alveolar macrophages have been presumed to be the important source of PAF in asthmatic subjects (48). Thus, the metabolic regulation of alkylacyl GPC in alveolar macrophages under physiological or pathological conditions seems to be an important point in connection with the biosynthesis and release of PAF. It has been considered that PAF is synthesized from alkylacyl GPC deposited in inflammatory and immunological cells through a deacylation-acetylation reaction (2). On the other hand, several investigators have demonstrated that 1-alkyl-2-acetyl-3-*sn*-glycerol was metabolized by cholinephosphotransferase to form PAF in microsomes from various rat tissues (52) and intact platelets (53,54). The high cholinephosphotransferase activity in macrophages seems also to be effective for the synthesis of PAF through the phosphotransferase pathway, although the enzymes catalyzing the transfer of CDP-choline to 1-alkyl-2-acetylglycerol and 1-alkyl-2-acyl(long chain) glycerol, respectively, were shown to be different in their sensitivity to dithiothreitol (14,52).

Taken together, it has been shown that both de novo synthesis and the remodeling of the fatty acids of either diacyl or alkylacyl GPC were very active in rabbit alveolar macrophages. The high cholinephosphotransferase activity and transacylation activity could account, at least in part, for the high formation of these phospholipids. The accumulation of arachidonic acid-containing alkylacyl GPC seems to be favorable for providing sufficient amounts of arachidonic acid and 1-alkyl-GPC (lyso PAF) for lipid mediator synthesis in this type of cell upon stimulation (3-7).

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METHODS

Rapid Separations of Diacyl- and Dialkylglycerol Enantiomers by High Performance Liquid Chromatography on a Chiral Stationary Phase

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Rapid and practical separations of 1,2- and 2,3-diacyl- and dialkyl-*sn*-glycerol enantiomers as their 3,5-dinitrophenylurethane derivatives were carried out by normal-phase high performance liquid chromatography on a chiral stationary phase, N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to γ-aminopropyl silanized silica. Complete separations of the racemates into enantiomers were achieved for both of the diacyl- and dialkylglycerols within 10 min using a stainless steel column (25 cm long) packed with the 5-μ particles, an isocratic elution with a mixture of hexane/ethylene dichloride/ethanol as a mobile phase and a UV detector. The *sn*-1,2-enantiomers were eluted ahead of the corresponding *sn*-2,3-enantiomers. Satisfactory separation of the *sn*-1,3-diacylglycerols from the corresponding enantiomers and the separation of the homologues differing in acyl and alkyl groups were also observed. The formations of hydrogen bonding and charge transfer complex between the urethane derivatives and the stationary phase may contribute to the enantiomer separations.
Lipids 22, 596–600 (1987).

A few studies have been published on identification and resolution of enantiomeric diacylglycerols. Bus et al. have resolved the signals from ester groups at the center of chirality of diacylglycerol acetates and butyrates by proton magnetic resonance spectroscopy in combination with a chiral reagent (1). Myher and Kuksis have resolved enantiomeric diacylglycerols via X-phosphatidylcholines and phospholipase C and have identified the individual molecular species of each enantiomer type by capillary gas liquid chromatography on a polar liquid phase (2). Optical rotatory dispersion and circular dichroism have also been commonly used for the analysis of enantiomeric acyl- and alkylglycerols (3,4). These techniques are powerful tools for the investigation on stereochemistry of acyl- and alkylglycerols, but are not simple or practical for obtaining the enantiomer composition of a mixture. Thus, there is considerable interest in chromatographic methods for the determination of enantiomeric acyl- and alkylglycerols (5).

The recent development of chiral stationary phases and chiral reagents for chromatography has permitted separations of various enantiomers (6). Michelsen et al. have reported the complete separations of mono- and dialkylglycerols and partial separation of diacylglycerols as their diastereomeric 1-(1-naphthyl)ethylurethane derivatives by high performance liquid chromatography (HPLC) on an achiral stationary phase (7). The incompletely separated diacylglycerol peaks could not be identified with optically pure samples. We have recently reported the enantiomer separations of long-chain monoacyl- (8), monoalkyl- (9) and diacylglycerols (10) as their 3,5-dinitrophenylurethane derivatives by HPLC on a chiral stationary phase, N-(*S*)-2-(4-chlorophenyl)isovaleroyl-D-phenylglycine bonded to silica gel. Although complete separations for monoacyl- and monoalkylglycerols were obtained within 15 min on the 25-cm long column, a longer column (75 cm long) and extremely long elution times (several hr) were required to obtain clear-cut separation for diacylglycerols on the same stationary phase.

Ôi and Kitahara have developed various chiral stationary phases for HPLC, which showed characteristic enantioselectivity for derivatives of amino acids, amines, carboxylic acids and alcohol enantiomers (11). We have found that some of these chiral phases also have enantioselectivity for diacyl- and dialkylglycerols as their 3,5-dinitrophenylurethane derivatives. This paper presents a rapid and practical HPLC method for the enantiomer separations of the long chain diacyl- and dialkylglycerol derivatives on a chiral stationary phase, N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine bonded to silica gel (11).

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MATERIALS AND METHODS

Samples. Synthetic 1,2-diacyl-*rac*-, 1,3-diacyl-*sn*- and 1,2-dialkyl-*rac*-glycerols used in this study (Table 1) were obtained from Sigma (St. Louis, MO), P.L. Biochemicals (Milwaukee, WI) and Bachem AG (Bubendorf, Switzerland), respectively. Prior to use, only 1,2-diacyl-*rac*-glycerol samples were purified by thin layer chromatography (TLC) on borate treated silica gel (12). Optically active 1,2- and 2,3-diacyl-*sn*-glycerols synthesized in our laboratory on the basis of the method of Howe and Malkin were also used (13).

The 3,5-dinitrophenylurethane derivatives were prepared by reaction of the samples of less than 1 mg with 3,5-dinitrophenyl isocyanate (Sumitomo Chemical Co., Osaka, Japan) of about 2 mg in dry toluene (4 ml) in the presence of dry pyridine (40 μl) at ambient temperature for 1 hr. The crude urethane derivatives of diacyl- and dialkylglycerols were purified by TLC on Silica Gel GF plates (20 × 20 cm, 0.25-mm thick, Analtech Inc., Newark, DE) using hexane/ethylene dichloride/ethanol (40:10:3, v/v/v and 40:10:1, v/v/v, respectively). Prior to use, the plates were activated at 110–120 C for 2 hr.

HPLC. HPLC separation was carried out with a Shimadzu LC-6A instrument equipped with a SCL-6A system controller (Shimadzu Co., Kyoto, Japan) and a chiral column (stainless steel, 25 cm × 4 mm i.d.) packed with 5-μ particles of N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically bonded to γ-aminopropyl silanized silica, Sumipax OA-4100 (Sumitomo Chemical). A Guard-pac precolumn module with a silica insert (Millipore Co., Milford, MA) was attached to the inlet of the chiral column. The analysis was done isocratically using a mixture of hexane/ethylene dichloride/ethanol (80:20:1, 150:20:1 or 250:20:1, v/v/v) as the mobile phase at a constant flow rate of 1 ml/min, which produced a pump pressure of about 500 psi. Prior to use, the mobile phase was filtered with a 2-μ PTFE membrane filter (Millipore). Usually, 1 μl of the samples dissolved in

TABLE 1

Chromatographic Data of Diacyl- and Dialkylglycerols as Their 3,5-Dinitrophenylurethane Derivatives on a Chiral Column, OA-4100

Acyl or alkyl		Mobile phase (hexane/ethylene dichloride/ethanol, v/v/v)								
		80:20:1 (A)			150:20:1 (B)			250:20:1 (C)		
Group	Position	Vr[a]	α[b]	Rs[c]	Vr	α	Rs	Vr	α	Rs
Dilauroyl	*sn*-1,3	7.06			24.55			65.77		
			1.07	0.72		1.07	0.89		1.10	2.31
	sn-1,2	7.57			26.24			72.53		
			1.15	2.16		1.15	2.33		1.12	1.91
	sn-2,3	8.70			30.13			81.39		
Dimyristoyl	*sn*-1,3	6.37			22.24			60.53		
			1.07	0.93		1.07	1.00		1.10	2.14
	sn-1,2	6.82			23.77			66.68		
			1.15	2.08		1.15	2.28		1.12	2.01
	sn-2,3	7.85			27.29			74.96		
Dipalmitoyl	*sn*-1,3	5.81			20.41			56.30		
			1.07	0.88		1.07	1.15		1.10	2.06
	sn-1,2	6.22			21.83			62.14		
			1.15	1.91		1.15	2.25		1.12	2.08
	sn-2,3	7.15			25.00			69.80		
Distearoyl	*sn*-1,3	5.37			18.94			52.84		
			1.07	0.84		1.06	1.12		1.10	2.03
	sn-1,2	5.74			20.14			58.26		
			1.15	1.89		1.15	2.25		1.13	2.04
	sn-2,3	6.61			23.12			65.95		
Dioleoyl	*sn*-1,3	5.99			21.30			56.64		
			1.04	0.60		1.02	0.60		1.11	2.00
	sn-1,2	6.24			21.76			63.01		
			1.15	2.02		1.17	2.23		1.12	1.92
	sn-2,3	7.18			25.36			70.40		
Dihexadecyl	*sn*-1,2	4.24			13.24			33.42		
			1.28	3.10		1.29	4.18		1.26	4.32
	sn-2,3	5.43			17.10			42.07		
Dioctadecyl	*sn*-1,2	3.90			12.27			35.73		
			1.28	2.84		1.29	3.98		1.26	4.39
	sn-2,3	4.99			15.60			44.98		

[a]Vr, retention volume (ml) corrected by subtracting the column void volume (2.68 ml).
[b]α, Separation factor (the ratio of the capacity ratios).
[c]Rs, peak resolution. $Rs = 2(t_1 - t_2)/(W_1 + W_2)$, where t = retention time (min) and w = peak width (min).

chloroform was injected into the column through a Rheodyne Model 7125 loop (20 μl) injector. Peaks were monitored at 0.02 AUFS with a Shimadzu SPD-6A variable wavelength (195–350 nm) UV detector having an 8-μl flow cell. Chromatograms, peak area percentages and retention times were obtained with a Chromatopac C-R3A (Shimadzu). The column void volume was determined by measuring the retention volume of pentane with a RI detector, Shodex Model SE-51 (Showa Denko Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Derivatives. Diacylglycerols react readily with 3,5-dinitrophenyl isocyanate without acyl migration in toluene solution in the presence of pyridine at ambient temperature. The resulting urethane derivatives have a sufficient absorption for HPLC detection over a wide range of UV wavelengths (8,10). The reaction of the dialkylglycerols with the isocyanate also proceeds quantitatively under the same conditions. The urethanes (Rf 0.63 for 1,2-dioctadecyl-*rac*-glycerol) were separated clearly from by-products (Rf 0 and 0.13), using hexane/ethylene dichloride/ethanol (40:10:1, v/v/v) as solvent. The by-products of Rf 0.13 appeared as a yellow band without UV irradiation. The Rf value of the corresponding diacylglycerol urethane derivatives in the same solvent system was somewhat lower—0.52. The faster mobility of dialkylglycerols without derivatization was also observed on Silica Gel TLC (5).

Separation. Figure 1 shows the HPLC chromatograms of optically active and inactive diacylglycerols as their 3,5-dinitrophenylurethane derivatives on a chiral column, OA-4100. Only the chromatogram of the racemate gave two clearly separated peaks with a 1:1 peak area ratio (Fig. 1A). The retention times of the former and later peaks (Fig. 1A) were in agreement with those of the *sn*-1,2- and *sn*-2,3-enantiomers (Figs. 1B and 1C), respectively. The identification of the peak components was also carried out by their complete overlapping in co-injection of the racemate with each enantiomer. Similar separation patterns were also observed for other racemic diacylglycerols used.

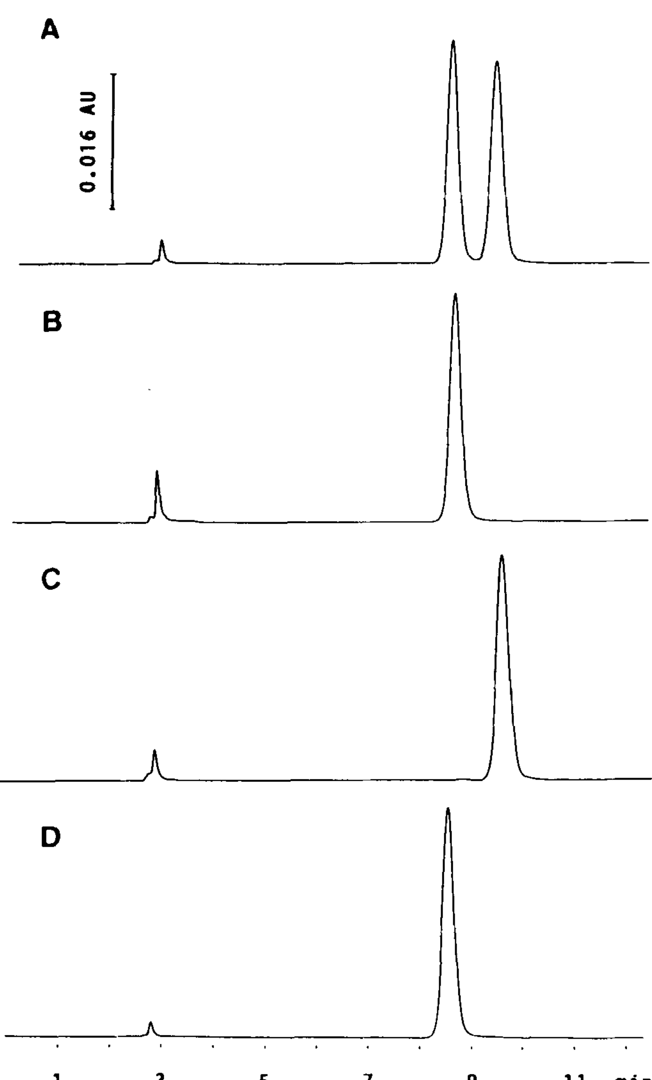

FIG. 1. Separation of diacylglycerol isomers as 3,5-dinitrophenylurethane derivatives on a chiral column, OA-4100. A, 1,2-dipalmitoyl-*rac*-glycerol; B, 1,2-dipalmitoyl-*sn*-glycerol; C, 2,3-dipalmitoyl-*sn*-glycerol; D, 1,3-dipalmitoyl-*sn*-glycerol. Mobile phase, hexane/ethylene dichloride/ethanol (80:20:1, v/v/v). Detection, 254 nm UV.

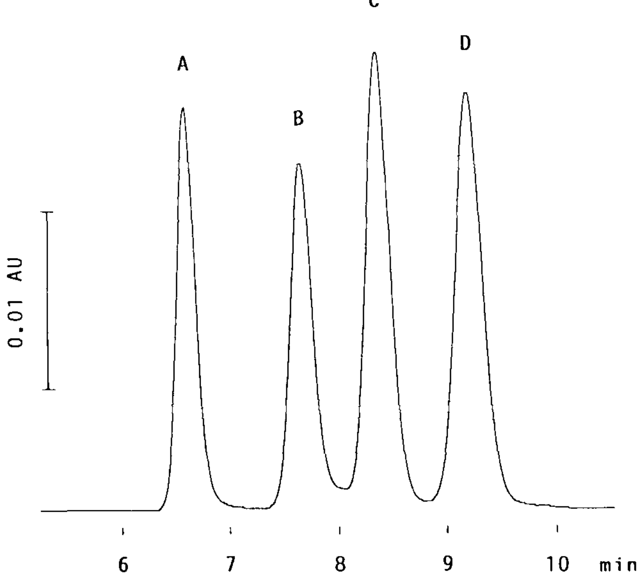

FIG. 2. Separations of dialkyl- and diacylglycerol enantiomers as 3,5-dinitrophenylurethane derivatives on a chiral column, OA-4100. A, 1,2-dioctadecyl-*sn*-glycerol; B, 2,3-dioctadecyl-*sn*-glycerol; C, 1,2-distearoyl-*sn*-glycerol; D, 2,3-distearoyl-*sn*-glycerol. Mobile phase, hexane/ethylene dichloride/ethanol (80:20:1, v/v/v). Detection, 254 nm UV.

As with diacylglycerol enantiomers, dialkylglycerol enantiomers were also separated completely with better resolution in a shorter elution time under the same conditions (Fig. 2). The faster elution of the dialkylglycerol urethane derivatives on the chiral silica column is probably due to their lesser polarity. The higher resolution of the dialkylglycerol enantiomers was also reported previously in the HPLC separation of the diastereomeric 1-(1-naphthyl)ethylurethane derivatives on an achiral silica column (7). By UV detection, each enantiomer of the racemic diacyl- and dialkylglycerols separated on OA-4100 showed a 1:1 peak area ratio at different wavelengths, which supports the effective separations of the racemic diacyl- and dialkylglycerols into their enantiomers without isomerization during the derivatization procedures and the HPLC analysis. Almost single peaks for 1,2-, 2,3- and 1,3-diacyl-*sn*-glycerols (Figs. 1B–1D) also support the absence of isomerization between the isomers. Thus, the chromatograms (Figs. 1 and 2) are characterized by complete separations of the racemates into the enantiomers within 10 min, with sharp and symmetrical peaks and faster elutions of *sn*-1,2-enantiomers than the corresponding *sn*-2,3-enantiomers. The faster elutions of the *sn*-1,2-enantiomers suggest stronger diastereomeric interactions with the stationary phase. In this study, a 0.2–0.5 nmol amount of the urethane derivatives was usually introduced into the column and was detected at 254 nm, a fixed wavelength in universal UV detectors. The detection at near 230 nm approximately doubled the sensitivity, but the peaks from the solvent chloroform, which appeared near 2.8 min, increased greatly.

The *sn*-1,3-isomers eluted just before the *sn*-1,2-enantiomers with partial separation (Fig. 1D) under the conditions used. This separation was improved by lengthening the elution time, which was obtained by reducing the mobile phase polarity (Fig. 3). The faster elution of the *sn*-1,3 isomer on the chiral silica column is mainly due to the lower polarity, because a similar observation for diacylglycerols was obtained in TLC on silica gel (5). The urethane derivatives of 2-monoacyl-*sn*-glycerols also showed a faster elution than those of 1-monoacyl-*rac*-glycerols on a chiral silica column, OA-2100 (8). The OA-4100 column used in this study showed 7,500 theoretical plates for the 2,3-dipalmitoyl-*sn*-glycerol peak. This value was reduced to 6,800 after 100 injections, but the enantiomers were still separated completely within 10 min.

Table 1 gives the HPLC data for isomeric diacyl- and dialkylglycerols as their 3,5-dinitrophenylurethane derivatives on OA-4100. The separations were examined using three mobile phases, hexane/ethylene dichloride/ethanol [(A) 80:20:1, (B) 180:20:1 and (C) 250:20:1, v/v/v],

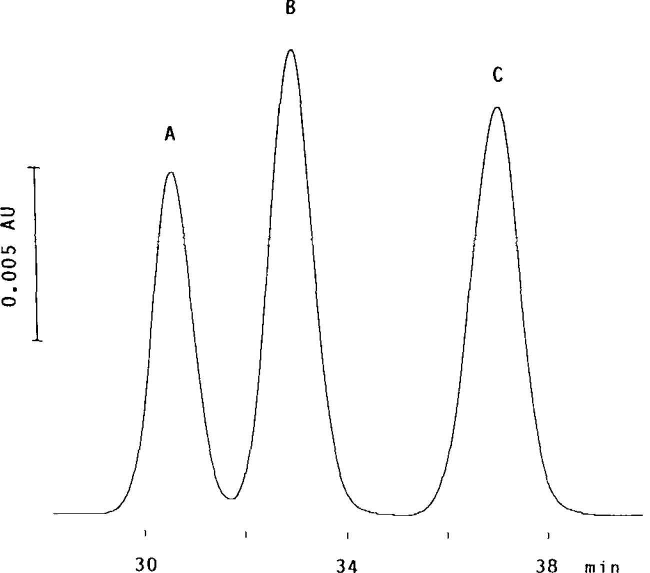

FIG. 3. Separation of 1,3-diacyl-*sn*-glycerols and the enantiomers as 3,5-dinitrophenylurethane derivatives on OA-4100. A, 1,3-dipalmitoyl-*sn*-glycerol; B, 1,2-dipalmitoyl-*sn*-glycerol; C, 2,3-dipalmitoyl-*sn*-glycerol. Mobile phase, hexane/ethylene dichloride/ethanol (150:20:1, v/v/v). Detection, 254 nm UV.

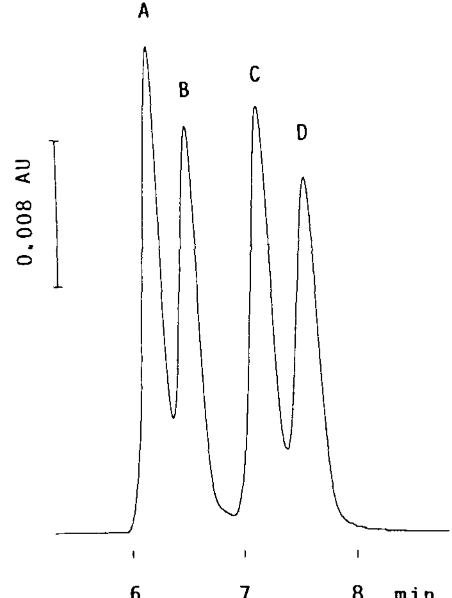

FIG. 4. Carbon number separation of dialkylglycerol enantiomers as 3,5-dinitrophenylurethane derivatives on a chiral column, OA-4100. A, 1,2-dioctadecyl-*sn*-glycerol; B, 1,2-dihexadecyl-*sn*-glycerol; C, 2,3-dioctadecyl-*sn*-glycerol; D, 2,3-dihexadecyl-*sn*-glycerol. Mobile phase, hexane/ethylene dichloride/ethanol (80:20:1, v/v/v). Detection, 254 nm UV.

which gave progressively longer elution times in order of A, B and C. In general, the elution times decrease with increasing polarity of the mobile phase in the normal-phase HPLC on the chiral silica columns. Of these mobile phases, mobile phase B gave the highest peak resolution for diacylglycerol enantiomers, as indicated by Rs values 2.2–2.3. On the other hand, mobile phase C gave the best separations for dialkylglycerol enantiomers (Rs 4.3–4.4) and for isomeric *sn*-1,3- and *sn*-1,2-diacylglycerols (Rs 2.0–2.3). Rapid and complete enantiomer separations for both of diacyl- and dialkylglycerols were achieved using mobile phase A.

In addition to the enantiomer separation, the OA-4100 column gives the carbon number separation of the acyl and alkyl groups and the separation of saturated and unsaturated species, which will be also caused by the silica gel support. Figure 4 shows the carbon number separation of the enantiomer homologues of dialkylglycerols on OA-4100 using mobile phase A. Apparently, the resolution of the peaks of the homologues differing by four alkyl carbons (Rs 1.1, not given in Table 1) is low compared with the resolution of the enantiomer peaks (Rs 2.8–3.1). The separation factor of the homologues differing by four alkyl carbons was 1.09 in each *sn*-1,2- and *sn*-2,3-enantiomer, as calculated from the data in Table 1. Almost equal values (1.08–1.11) were also obtained for those of the diacylglycerol homologues. Figure 5 shows the separation of saturated and unsaturated diacylglycerol enantiomers on OA-4100. As with the saturated diacylglycerols, the dioleoylglycerol racemate was also separated completely within 10 min into the enantiomers with almost equal separation factor 1.15 and peak resolution 2.0, using mobile phase A. The separation factors 1.09–1.11 of the diacylglycerols differing by two double bonds, that is, distearoyl- and dioleoylglycerols, are almost equal to those 1.08–1.11 of the diacylglycerols differing by four

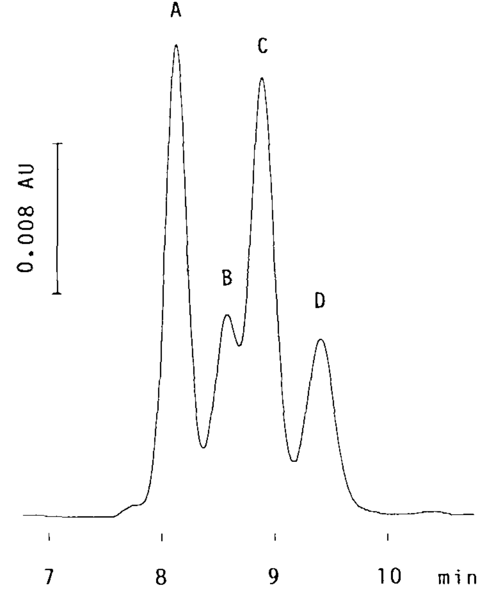

FIG. 5. Separation of saturated and unsaturated diacylglycerol enantiomers as 3,5-dinitrophenylurethane derivatives on OA-4100. A, 1,2-distearoyl-*sn*-glycerol; B, 1,2-dioleoyl-*sn*-glycerol; C, 2,3-distearoyl-*sn*-glycerol; D, 2,3-dioleoyl-*sn*-glycerol. Mobile phase, hexane/ethylene dichloride/ethanol (80:20:1, v/v/v). Detection, 254 nm UV.

acyl carbons, such as dipalmitoyl- and distearoylglycerols. Thus, the HPLC of a mixture of saturated and unsaturated diacylglycerols on the chiral column gives complex chromatograms. Therefore, preliminary separations by reversed-phase HPLC, argentation TLC and others are necessary to obtain accurate enantiomer composition of a mixture of saturated and unsaturated species.

```
        O (S)     O   (R)
        ||  *     ||   *
—O\ |
   Si—(CH2)3—N—C—CH—N—C—N—CH—(naphthyl)
—O/           H    |   H     H  |
                  CH           CH3
                 /  \
              CH3    CH3
```

FIG. 6. Chemical structure of the OA-4100 stationary phase.

Figure 6 shows the structure of the OA-4100 stationary phase. This phase contains a chiral urea group and a chiral 1-(α-naphthyl)ethyl group, which have the abilities to serve either as a donor or an acceptor in hydrogen bonding and to serve as a π-donor, respectively (11). Thus, the diastereomeric hydrogen bonding association and π-π donor-acceptor interaction between the stationary phase and the solutes may contribute to the enantiomer separations of diacyl- and dialkylglycerols. In comparison with OA-2100 used previously (8–10), OA-4100 has two more NH groups in the molecule, which may facilitate the hydrogen bonding association with the carbonyl groups in the solutes. The introduction of an NH group as the urethane derivatives into diacyl- and dialkylglycerols may also contribute to the excellent enantiomer separations. In addition, the naphthyl group in OA-4100 may form more easily the charge transfer complex with the 3,5-dinitrophenyl group in the urethane derivatives than the chlorophenyl group in OA-2100.

ACKNOWLEDGMENT

We are grateful to Dr. N. Ôi for useful suggestions on chiral columns.

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[Received March 6, 1987]

ERRATUM

Two figures were inadvertently transposed in the June issue of *Lipids*. The figure which appeared over the Figure 4 legend, on page 405, should have been over the Figure 5 legend, on page 406, and the one over the Figure 5 legend should have appeared over the Figure 4 legend. The error occurred in "Lipid Peroxidation in Erythrocyte Membranes: Cholesterol Product Analysis in Photosensitized and Xanthine Oxidase-Catalyzed Reactions," by Albert W. Girotti, Gary J. Bachowski and John E. Jordan, published in *Lipids 22*, 401–408 (1987).

```
        O (S)      O   (R)
 —O  |   ||  *      ||   *
   > Si—(CH2)3—N—C—CH—N—C—N—CH—(naphthyl)
 —O          H      |  H     H  |
                    CH          CH3
                   /  \
                 CH3  CH3
```

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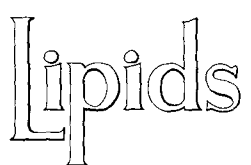

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Lipid Composition and Amino Acid Transport in a Nystatin-Resistant Mutant of *Aspergillus niger*

Chitra Mazumder, Manikuntala Kundu, Joyoti Basu and **Parul Chakrabarti***
Department of Chemistry, Bose Institute, Calcutta, India

A nystatin-resistant mutant of *Aspergillus niger* has been isolated and used as a model system to study the effect of altered sterol levels on lipid composition, transport behavior and physical properties of membrane lipids. There is a decrease in the sterol to phospholipid ratio in the mutant compared to the wild type. Although there is no qualitative change in phospholipid composition, the mutant contains a higher amount of phosphatidylcholine and a lower amount of phosphatidylethanolamine compared to the wild type. The most significant change is the elevated level of linoleic acid in the mutant, concomitant with a decreased level of oleic acid. These adaptive changes to nystatin resistance are manifested in the altered thermotropic behavior of membrane lipids as studied by the steady-state fluorescence polarization technique. These changes are also associated with altered membrane permeability as evidenced by the change in V_{max} values for uptake of some amino acids in the mutant compared to the wild type.
***Lipids 22,* 609–612 (1987).**

Polyene antibiotics have been shown to interact with membrane sterols of fungal cells (1,2). Various polyene antibiotics such as nystatin and amphotericin B have been used to get resistant strains of fungi containing low levels of sterols (3,4). However, detailed studies on the interrelationships among altered sterol content, overall lipid composition, physical properties of membranes and transport phenomena in fungi remain to be carried out. In the present study, a nystatin-resistant mutant of *Aspergillus niger* has been isolated and used as a model system to study some of the effects of altered sterol level on lipid composition, physical properties and functions of membranes. The results of our present studies with this mutant suggest that, concomitant with altered sterol level, there is overall modulation in the phospholipid and fatty acid composition of the mutant. These alterations in membrane lipids may be responsible for the altered rates of uptake of various amino acids in the mutant compared to the wild type *A. niger.*

MATERIALS AND METHODS

Materials. Nystatin, 1,6,diphenyl-1,3,5-hexatriene (DPH) and standard lipids were purchased from Sigma Chemical Co. (St. Louis, MO). Standard fatty acid methyl esters were purchased from Nu-Chek-Prep (Elysian, MN); Silica Gel G and Silica Gel H were from E. Merck (Darmstadt, FRG); and 1,6-ditertiarybutyl-p-cresol was from Aldrich Chemicals (Milwaukee, WI). All ^{14}C-labeled amino acids were obtained from the Bhaba Atomic Research Centre, Trombay, India. All other solvents and reagents used were of analytical grade.

Growth of organism. Wild-type *A. niger* V_{35} was collected from the stock culture of the Department of Microbiology of the Bose Institute. Stock cultures of V_{35} were grown at 28 C on solid agar slants of Czapek-Dox medium (5) containing the following (g/l of distilled water): glucose, 40.0; $NaNO_3$, 2.0; KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; and $ZnSO_4 \cdot 7H_2O$, 0.01. Media for slants were solidified with 2% (w/v) agar.

Isolation of a nystatin-resistant mutant. Nystatin-resistant cells were obtained according to the method of Nobre et al. (6), where cells were grown in the presence of increasing concentrations of nystatin. The inoculum was a suspension of conidia at a concentration of 10^7 cells/ml. Resistance was induced by serial transfer at the following drug concentrations (U/ml): 11, 22, 44, 88, 176, 352, 704, 1408, 2816, 5632 and 8550. The minimum inhibitory concentrations of the wild type and the nystatin-resistant mutant were found to be 17 U/ml and 8550 U/ml, respectively. A mutant 500-fold more resistant to nystatin than the wild type was obtained.

Isolation of conidia. Conidial cell suspensions were prepared by harvesting conidia into sterile distilled water and filtering through glass-wool to remove mycelial fragments. The cell suspensions were then centrifuged at 30,000 × g to pellet down the conidia, which were washed thoroughly with excess distilled water and lyophilized.

Extraction of lipids. Lipids were extracted from washed and freeze-dried cells following the method of Folch et al. (7). Antioxidant 2,6-ditertiary butyl-p-cresol (50 mg/l) was added to the solvents to prevent oxidation. Lipids were stored in chloroform at −20 C in the presence of nitrogen.

Lipid analysis. Neutral and polar lipid fractions were separated by preparative thin layer chromatography (TLC) using Silica Gel G and acetone as developing solvent. Individual phospholipids were separated by preparative TLC on Silica Gel H according to the method of Skipski et al. (8) using the solvent system chloroform/methanol/acetic acid/water (25:15:4:2, v/v/v/v). Individual components were extracted and the phosphorus content was estimated according to the method of Ames and Dubin (9). Neutral lipid extracts were fractionated by TLC as described previously (10) using the solvent system petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v). Free sterol was eluted with chloroform/methanol (4:1, v/v) and estimated colorimetrically by the method of MacIntyre and Ralston (11) using ergosterol as standard.

Fatty acid analyses. Fatty acids isolated from phospholipids obtained from the conidia of both the wild type and the mutant were analyzed by gas liquid chromatography. Phospholipids were hydrolyzed, and methyl esters were prepared from the liberated free fatty acids by the method of Luddy et al. (14). These were identified against appropriate standards and quantitated by electronic integration on a 15% diethylene glycol succinate column (3 mm id × 1.5 m), using nitrogen at a flow

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Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; TLC, thin layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin.

rate of 50 ml/min at an operating temperature of 170 C on a Shimadzu(GCR1A) gas chromatograph fitted with a dual flame ionization detector and an automatic data processor RPRG1.

Liposome preparation. Phospholipids and sterols extracted from the different systems were mixed in the molar ratio in which they were present in each of these systems. Desired lipid mixtures and DPH were dissolved in chloroform at a lipid to probe ratio of 750:1. A thin film was made under nitrogen and then dried under vacuum for 4 hr. The mixture was resuspended in 10 mM phosphate buffer, pH 6.0, and sonicated in a water-bath sonicator (12). The final DPH concentration was 1 μM.

Steady-state fluorescence pllarization. Fluorescence polarization was measured in a Perkin-Elmer fluorescence spectrophotometer (excitation wavelength, 360 nm; emission wavelength, 430 nm). Polarization values (P) were determined according to ref. 13:

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the intensities of parallel and perpendicular polarized light, respectively. These were calculated according to the above relation. The standard deviation of each polarization value was 0.002. Temperatures were maintained within ± 0.5 C. Since the contribution due to scattering determined using lipid samples of identical composition without DPH was found to be less than 2% of total emitted light at 430 nm, no correctons were made.

Transport assay. Cells were grown in solid Czapek-Dox medium as described before. Conidia were isolated, washed and suspended in 1 ml of 10 mM phosphate buffer (pH 6.0) at concentration of 4×10^7 cells/ml. Uptake was started by the addition of different concentrations of ^{14}C-labeled amino acid (50 μCi/μmol) and stopped after 2 min by adding 10 ml of ice-cold water. Two min were found to be within the linear range of uptake. The diluted suspension was rapidly filtered through 0.45 μM Millipore filter (HAWP 025 00), and the filter was dried and counted in an LKB liquid scintillation counter (Mini Beta 1211) using a toluene-based scintillation fluid.

Statistical analysis. Student's t-test was used to assess levels of significance. Values of $P < 0.01$ were considered to be significant.

RESULTS

Lipid analyses. Since phospholipids and sterols are the major lipid components modulating the properties of biomembranes, these were analyzed in the conidia of both the wild type and the nystatin-resistant mutant of *A. niger.* The free sterol contents were found to be 5.6% and 3.6% of the total lipid of the wild type and the mutant, respectively. There was no qualitative change in phospholipid composition in the conidial lipids of the mutant compared to the wild type. However, the amount of phosphatidylethanolamine (PE) was found to decrease in the mutant, concomitant with an increase in phosphatidylcholine (PC) (Table 1). Phosphatidylinositol (PI) and phosphatidylserine (PS) could not be separated using the solvent system described.

Analysis of the fatty acid of the total phospholipid of the mutant and the wild type revealed significant alteration of fatty acid composition. The phospholipids of the mutant were found to contain elevated levels of linoleic acid (18:2) compared to the wild type. The level of oleic acid (18:1) was, on the other hand, lower in the mutant than in the wild type (Table 2).

Fluorescence polarization studies. These were carried out with liposomes prepared from isolated conidial phospholipids and sterols of both the mutant and the wild type. A discontinuity in the fluorescence polarization plots was observed at 18 C in the case of the mutant, while no discontinuities were observed in the case of the wild type (Fig. 1).

Amino acid transport. In the present study, uptake of the amino acids leucine, lysine, glutamic acid and aspartic acid was studied in the wild type and the nystatin-resistant mutant of *A. niger.* From the Lineweaver-Burk plots, a comparison of the K_m values for the uptake of a particular amino acid showed no significant differences between the mutant and the wild type (0.16 mM for aspartic acid, 0.2 mM for glutamic acid, 0.2 mM for lysine and 0.13 mM for leucine) (Fig. 2). However, V_{max} values for the uptake of some amino acids showed differences between the two types of cells. The V_{max} values for uptake of aspartic acid, lysine and leucine were higher in the mutant than the wild type (Table 3), while there was no significant change in the V_{max} values for glutamic acid uptake.

TABLE 1

Lipid Composition of the Conidia of Wild Type *A. niger* and Its Nystatin-Resistant Mutant

Strain	Phospholipid (μg/g dry weight)				PC/PE (ratio)	Sterol/phospholipid (μmol/μmol)
	PC	PE	CL	PI + PS		
Wild	2.5 ± 0.12	2.92 ± 0.16	0.42 ± 0.21	0.75 ± 0.32	0.86	0.83
Mutant	2.85 ± 0.21	1.76 ± 0.13	0.26 ± 0.11	0.64 ± 0.26	1.62	0.6

PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin; PI, phosphatidylinositol; PS, phosphatidylserine. Determinations of lipid phosphorus and identification of different phospholipids were performed as described in Materials and Methods. Phosphorus contents were multiplied by 25 to obtain content of the total phospholipids. In the case of cardiolipin, this value was further divided by 2. Values are mean ± S.D. of five different sets of determinations.

TABLE 2

Fatty Acid Composition of Phospholipids of the Conidia of Wild Type and Nystatin-Resistant *Aspergillus niger*

Fatty acid	% of total	
	Wild	Mutant
16:0	25.72 ± 0.52	25.45 ± 0.43
18:0	3.35 ± 0.29	3.38 ± 0.32
18:1	31.85 ± 0.39*	25.69 ± 0.31*
18:2	31.57 ± 0.51**	37.64 ± 0.46**
18:3	1.04 ± 0.37	1.97 ± 0.35

Fatty acid analysis was performed as described in Materials and Methods. Individual fatty acids present in amounts less than 1% of the total fatty acids have not been shown. Values are mean ± S.D. of five sets of determinations. *, P < 0.001; **, P < 0.001.

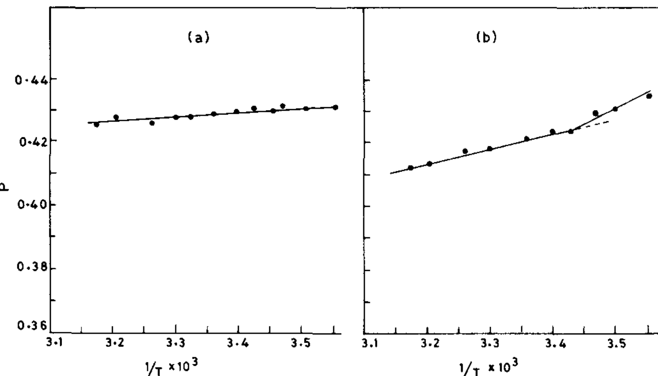

FIG. 1. Fluorescence polarization plots of liposomal membranes of *A. niger*: (a) wild type, (b) nystatin-resistant mutant. Fluorescence polarization measurements were carried out as described in Materials and Methods.

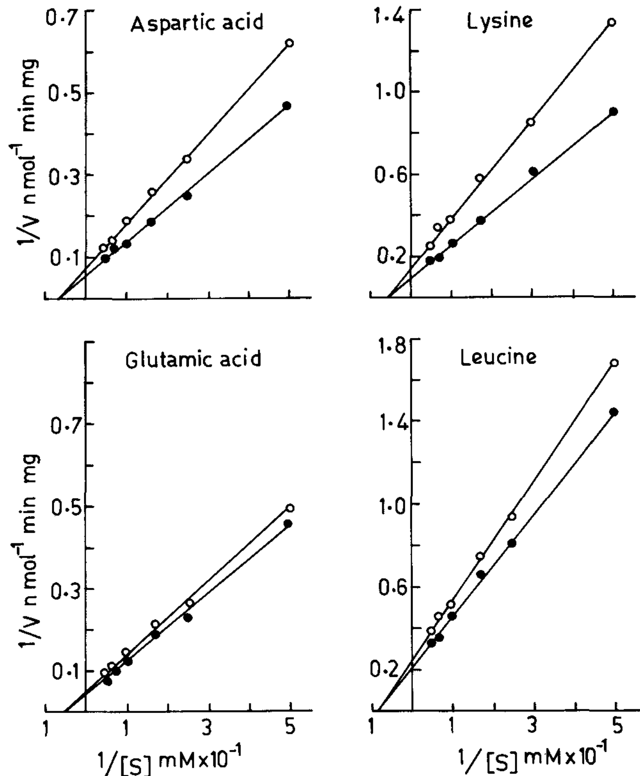

FIG. 2. Various concentrations of each amino acid were prepared using a specific activity of 50 μCi/μmol. Transport assay was carried out as described in Materials and Methods. Transport rates have been expressed per mg dry weight of cells for the wild type (○) and the nystation-resistant mutant (●).

DISCUSSION

Sterols have long been recognized as important membrane constituents responsible for modulating the functions of eukaryotic membranes (15,16). However, the precise way in which they regulate the physical properties and biochemical functions of cell membranes still remains to be understood. In the present case, a nystatin-resistant mutant of *A. niger* was isolated. The sterol/phospholipid molar ratio is lower for the conidial lipids of the mutant compared to the wild type. Some of the effects of altered sterol content have been reported here. The overall phospholipid composition suggests that the mutant adapts to a certain extent to changes in the sterol content by altering levels of individual phospholipid species. The observed increase in PC content and simultaneous decrease in PE content in the mutant compared to the wild type is one of the responses of *A. niger* to adaptation to growth in the presence of nystatin. Even more significant than the change in phospholipid composition is the altered fatty acid composition of the mutant. Studies with the yeast *Saccharomyces cerevisiae* have also shown that exogenous sterol modulates yeast plasma membrane phospholipid and fatty acid composition (17).

TABLE 3

V_{max} Values for Uptake of Some Amino Acids in *A. niger* V_{35} and Its Nystatin-Resistant Mutant

Amino acid	V_{max} (nmol/min/mg)	
	Wild	Mutant
Leucine	4.16 ± 0.25*	5 ± 0.16*
Lysine	6.25 ± 0.32*	10 ± 0.21*
Glutamic acid	20 ± 0.32^{NS}	20 ± 0.22^{NS}
Aspartic acid	12.5 ± 0.12*	16.5 ± 0.16*

Transport assay was carried out as described in Fig. 2. Values are mean ± S.D. of five independent determinations. NS, Nonsignificant; *, P < 0.01.

The nature of the fatty acyl chain of phospholipids is one of the most important factors modulating membrane fluidity and integrity. The polarization values decreased with increasing temperature, indicating an increase in probe mobility with increasing temperature. The slopes of the plots show that the rate of this decrease with temperature was higher in the case of the mutant, suggesting increased relative mobilities of lipids (Fig. 1). This is consistent with the increased level of unsaturation and decreased level of sterol in the mutant compared to the wild type. Liposomes of the wild type show no

discontinuity in plots of fluorescence polarization. However, liposomes of the mutant show physical changes in membrane lipids characterized by a discontinuity in fluorescence polarization plots, suggesting a gel-liquid crystalline transition. Compensatory changes in the fatty acyl chains of phospholipids, associated with sterol depletion, have been shown in LM-cell sterol mutants (18). Such depletion leads to decrease in the high melting phospholipids in the plasma membrane of LM-cells. Our findings suggest that such an adaptation is necessary in the present case also to preserve essential membrane functions and growth of the organism. It will also be interesting to see whether changes in fatty acid composition are related to any qualitative changes in sterols in the nystatin-resistant mutant. The effects of supplementing LM-cell mouse fibroblasts with different cholesterol analogues have shown that secondary changes in fatty acid composition occur, probably to prevent phase separation, since different sterols partition to varying extents among different phospholipid domains (19-21). However, it appears from the present results that there is a coordinated regulation of sterol, phospholipid and fatty acid composition in *A. niger* that is reflected in the altered physical properties of the conidial lipids of the nystatin-resistant mutant.

A preliminary effort to explore the effect of altered lipid composition as a result of sterol depletion on the biochemical functions of the mutant was made by studying the transport of two acidic, one neutral and one basic amino acid in both the wild type and the mutant. Our results show that the transport of different amino acids is affected to different extents by altered sterol content and overall changes in lipid composition associated with nystatin-resistance in *A. niger.* Since different permeases are known to be responsible for uptake of different amino acids in *Aspergillus* species (22-24), results of the present study suggest that these permeases, by virtue of occupying different microenvironments in the lipid bilayer, are affected to different extents by changes in lipid composition. However, in general, there is a change in membrane permeability as evidenced from the increased V_{max} values for uptake of lysine, leucine and aspartic acid in the mutant as compared to the wild type. These results agree with the findings of several workers with both artificial and natural cellular membranes that altered sterol levels lead to a change in membrane permeability and in membrane-associated functions such as the activities of membrane-bound enzymes (25-29).

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Interaction of (n-3) and (n-6) Fatty Acids in Desaturation and Chain Elongation of Essential Fatty Acids in Cultured Glioma Cells

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Recent research in various biological systems has revived interest in interactions between the (n-6) and (n-3) essential fatty acids. We have utilized cultured glioma cells to show that linolenic acid, 18:3(n-3), is rapidly desaturated and chain elongated; 20:5(n-3) is the major product and accumulates almost exclusively in phospholipids. We examined effects of various (n-6), (n-3), (n-9) and (n-7) fatty acids at 40 μM concentration on desaturation and chain elongation processes using [1-^{14}C]18:3(n-3) as substrate. In general, monoenoic fatty acids were without effect. The (n-6) fatty acids (18:2, 18:3, 20:3, 20:4 and 22:4) had little effect on total product formed. There was a shift of labeled product to triacylglycerol, and in phospholipids, slightly enhanced conversion of 20:5 to 22:5 was evident. In contrast, 22:6(n-3) was inhibitory, whereas 20:3(n-3) and 20:5(n-3) had much less effect. At concentrations <75 μM, all acids were inhibitory. Most products were esterified to phosphatidylcholine, but phosphatidylethanolamine also contained a major portion of 20:5 and 22:5. We provide a condensed overview of how the (n-6) and (n-3) fatty acids interact to modify relative rates of desaturation and chain elongation, depending on the essential fatty acid precursor. Thus, the balance between these dietary acids can markedly influence enzymes providing crucial membrane components and substrates for biologically active oxygenated derivatives.
Lipids 22: 613-619 (1987).

In recent years, there has been a renewed interest in linolenic acid [18:3(n-3)] and its metabolites and in the interactions between the (n-3) and (n-6) families of fatty acids in various biological systems (1-3). In particular, the (n-3) fatty acids that constitute a major portion of the polyunsaturated fatty acids in fish oils have been shown to have potential influences on lowering levels of circulating lipids (1-7), on platelet aggregation (4,8,9) and on the cardiovascular system in general (1,4,10). Much of this interest has arisen from studies with Greenland Eskimos, in which a correlation has been indicated between a lower incidence of coronary heart disease and longer bleeding times and their relatively high consumption of (n-3) fatty acids from marine sources compared to control populations (3,11,12). Recent evidence supports potential interaction of (n-3) acids with (n-6) fatty acids influencing the immune system (1,13) and tumor incidence and growth (14-16).

The relatively high levels of (n-3) acyl chains in the central nervous system, retina and testes (17) support a specific role for these acids in such specialized tissues. The (n-3) are less effective than (n-6) fatty acids in altering classical signs of essential fatty acid deficiency (1,2), indicating that they are not equivalent in their actions. A human case ascribed to deficiency of 18:3(n-3) has been reported (19), although concern has been expressed as to whether this was specific to the (n-3) acids (20,21).

On both nutritional and metabolic levels, much attention has focused on competition between the (n-3) and (n-6) families and resulting alterations in the products formed from them. Competition between (n-3) and (n-6) fatty acids for desaturation and chain elongation steps has been described in in vivo (22-25) and in vitro (25-30) experiments. The (n-3) acids, particularly eicosapentaenoic acid [20:5(n-3)], can compete with 20:4(n-6) in eicosanoid (e.g., prostaglandin and thromboxane) formation (31,32), can form products with different biological properties (e.g., leukotrienes) (33), or can produce products (e.g., prostacyclin I_3) with similar activating properties (10).

In previous studies, we used neuroblastoma and glioma cells in culture to examine the metabolism and interaction of (n-3) and (n-6) fatty acids in an experimental model system that can be carefully modulated and controlled. Both cell lines have the capacity for Δ6 and Δ5 desaturation and the alternating chain elongation steps involved in metabolism of these acids. With 18:2(n-6), the major products are 20:3(n-6) and 20:4(n-6), and with 18:3(n-3) the major product is 20:5(n-3), with lesser amounts of 20:4(n-3) and 22:5(n-3). No 22:6(n-3) (docosahexaenoic acid) is formed due to an absence of Δ4 desaturase in these transformed cells. We have described the relative metabolism and distribution of the substrates and products in intracellular lipids (34), major differences in the alteration of 18:2(n-6) metabolism by various unlabeled fatty acids (35), some aspects of the mechanism of activation of 18:2(n-6) metabolism (36) and the kinetics of the subcellular distribution and turnover of 18:2(n-6) (37).

In the present study, we examined desaturation, chain elongation and esterification of 18:3(n-3) and its derivatives and the effects of a variety of monoenoic, (n-3) and (n-6) fatty acids on these processes. These results have been combined with previous observations to provide a condensed overview of the complex interactions between these two families of essential fatty acids.

MATERIALS AND METHODS

Materials. All chemicals and reagents used were of the finest grade obtained from commercial suppliers. [1-^{14}C]-18:3(n-3) (55 mCi/mmol) and [1-^{14}C]18:2(n-6) (59 mCi/mmol) were purchased from DuPont Canada Inc. (NEN Products, Lachine, Quebec). Purity of these acids, determined by several analytical procedures described previously (38), was >97%. Nonradioactive fatty acids, lysophosphatidylcholine and other lipid standards were purchased from Serdary Research Laboratories (London, ON), Sigma Chemical Co. (St. Louis, MO) and Supelco (Bellefonte, PA). [^{3}H-Methyl]lysophosphatidylcholine, obtained as a by-product of synthesis of [^{3}H-methyl]sphingomyelin, was purified and characterized (>97.5% pure) using replicate

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thin layer chromatographic (TLC) development as previously described (39). Fatty acid solutions (20 mg/ml) were maintained under N_2, and purity was monitored by gas liquid chromatography (GLC) of methyl esters. All solvents were high performance liquid chromatography grade from Fisher.

Glioma cell cultures. C-6 glioma cell lines were maintained in 150-cm² flasks (Corning Glass Works, Corning, NY) in 45 ml Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco Canada Ltd., Burlington, ON) and penicillin: streptomycin solution (100 units and 100 μg/ml, respectively) (35). Cells were maintained at 37 C in a humidified atmosphere of 95% air/5% CO_2 and were subcultured every 5–7 days. For incorporation and metabolism studies, cells were transferred to 60-mm diameter dishes at a density of 0.5–1.0 × 10^6 cells in 5 ml medium. After 72 hr the medium was removed and replaced with 2.0 ml Dulbecco's modified Eagle's medium without fetal bovine serum. After another 24 hr, mixtures of [1-^{14}C]fatty acids and unlabeled fatty acid suspended by sonication at 37 C in sterile 5% bovine serum albumin were added in a 100-μl volume to give a final fatty acid concentration of 2 μM (2 × 10^6 dpm) for the [1-^{14}C]fatty acid and 40 μM for the unlabeled fatty acids (unless otherwise specified). Cells were incubated for 2 or 4 hr at 37 C.

Extraction and analysis of lipids. Details of the extraction and analyses procedures have been published previously (35,37,40,41). In brief, after incubation the cells were harvested, washed with phosphate-buffered saline and centrifuged. An aliquot was removed for analysis of protein by the method of Lowry et al. (42) with bovine serum albumin as standard; an aliquot of medium and cell washes were counted for total medium radioactivity. The cell pellet was extracted and radioactivity in an aliquot of extract quantitated by liquid scintillation counting. The lipid extract was separated into neutral lipid and phospholipid fractions by silicic acid chromatography and further resolved by TLC (41). Fatty acyl methyl esters were separated and quantitated using radio-GLC and a radioactivity flow monitor as described (35,43).

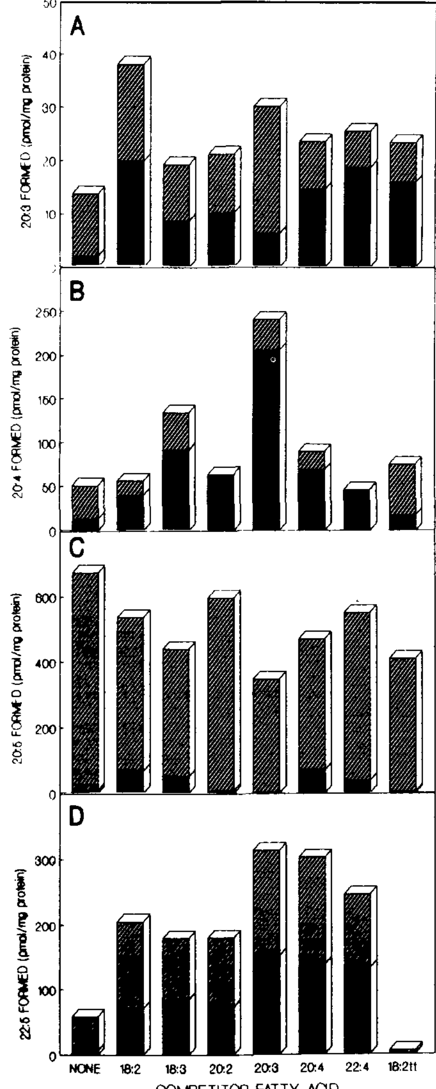

FIG. 1. Influence of various unlabeled (n-6) fatty acids on the formation of individual products by desaturation and chain elongation of [1-^{14}C]18:3(n-3) in cultured glioma cells. Cells were incubated for 2 hr with 0.5 μCi [1-^{14}C]18:3(n-3) and 40 μM unlabeled fatty acid as indicated under each bar along the horizontal axis. For controls no exogenous unlabeled fatty acid was added. Cells were extracted and lipids were fractionated on silicic acid columns. Neutral lipid and phospholipid fractions were transesterified and fatty acid methyl esters were analyzed by radio gas liquid chromatography. (A) Label in 20:3; (B) label in 20:4; (C) label in 20:5; (D) label in 22:5. Solid bar, neutral lipid; shaded bar, phospholipid. Values are the mean for the following replicate experiments: control (none), n = 14; 20:4(n-6), n = 10; 18:2(n-6), n = 8; all others, n = 4. In the fatty acid abbreviations, the number before the colon designates the number of carbon atoms in the acyl chain and the number following the colon indicates the number of double bonds. The (n-X) nomenclature designates the position of the first double bond from the methyl end (for more details see ref. 44); t indicates *trans* double bonds; all others are *cis* double bonds.

RESULTS

Effects of various fatty acids on 18:3(n-3) metabolism. When C-6 glioma cells were incubated with 2 μM [1-^{14}C]18:3(n-3), the labeled fatty acid was rapidly incorporated into the cell; uptake within 2 and 4 hr was 69% and 81% of total incubated fatty acid, respectively. In the neutral lipid fraction, >95% of the label was in triacylglycerol, and free fatty acid accounted for <2% of the label. As previous studies with [1-^{14}C]18:2(n-6) as substrate (35,36) had shown that 40 μM of stimulatory fatty acids gave maximal increase in desaturation, chain elongation and esterification, fatty acids were examined at 40 μM for their effect on metabolism of [1-^{14}C]18:3(n-3). Unlabeled fatty acid reduced total uptake of label by 5–20% at 2 hr, but by 4 hr consistently gave <10% reduction. All unlabeled fatty acids produced a greater incorporation into the neutral lipid fraction.

When competing unlabeled fatty acids were grouped [i.e., (n-6) acids compared to (n-3) acids], distinctly different effects on the metabolism of [1-^{14}C]18:3(n-3) were noted (summation of all products in Figs. 1 and 2). The (n-6) fatty acids gave very little change in total product formed, although there was greater esterification to triacylglycerol. An exception was the *trans,trans*-isomer of 18:2(n-6), which reduced product formation and esterification to phospholipid by >30%. Of the (n-3) acids

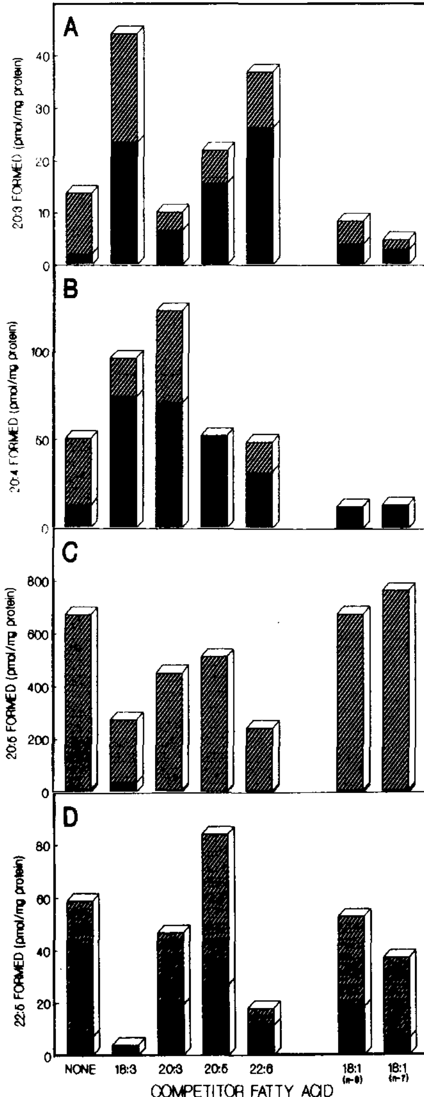

FIG. 2. Influence of various (n-3) and monoenoic fatty acids on the formation of individual products by desaturation and chain elongation of [1-^{14}C]18:3(n-3). Fatty acid methyl esters were obtained and analyzed as described for Fig. 1. (A) Label in 20:3; (B) label in 20:4; (C) label in 20:5; (D) label in 22:5. Solid bar, neutral lipid; shaded bar, phospholipid. Values are the mean for the following replicate experiments: control (none), n = 14; 18:3(n-3) and 22:6(n-3), n = 6; all others, n = 4.

tested, 18:3(n-3) and 22:6(n-3) gave the greatest reduction. [For comparison, unlabeled 18:3(n-3) has been treated as any other competitor, although "substrate or isotope dilution" is primarily responsible for the greater esterification of unaltered labeled substrate to neutral lipid and an overall reduction in product formation.] The 20:3(n-3) and 20:5(n-3) fatty acids were less inhibitory to product formation, but did reduce the amount of product esterified to phospholipid by >25%. The amount of product esterified to triacylglycerol was less in the presence of (n-3) acids than with (n-6) acids. 18-Carbon monoenoic isomers [oleic acid, 18:1(n-9), and *cis*-vaccenic acid, 18:1(n-7)] had very little influence on product formation and relative esterification.

The effects of various unlabeled fatty acids on the accumulation and esterification of individual products from [1-^{14}C]18:3(n-3) are shown in Figure 1 [unlabeled (n-6) fatty acids] and Figure 2 [unlabeled (n-3) fatty acids, 18:1(n-9) and 18:1(n-7)]. Labeled 20:5(n-3) accounted for >85% of the product formed in control incubations and even for the most altered condition accounted for >50% of total product. This major product was esterified almost exclusively to phospholipid. All (n-6) and (n-3) competitor acids reduced accumulation of labeled 20:5(n-3); exceptions were the two monoenoic isomers. The second most abundant labeled product was 22:5 formed by chain elongation of labeled 20:5. Except for *trans,trans*-18:2(n-6), which was markedly inhibitory to labeled 22:5 formation, all (n-6) acids increased formation of 22:5 and its esterification to both phospholipid and neutral lipid, particularly the latter. Accumulation of other labeled products (20:3 and 20:4) was altered variably. Labeled 20:3 was increased by all (n-6) acids, but at maximal stimulation this accounted for <4% of total labeled product. Unlabeled 20:3(n-6) gave a major increase in the accumulation of labeled 20:4 in the neutral lipid fraction.

The unlabeled (n-3) acids all reduced accumulation of labeled 20:5 in phospholipids (Fig. 2). Formation and esterification of labeled 22:5 to neutral lipid increased with all unlabeled (n-3) acids. All (n-3) acids gave increased levels of labeled 20:3 and 20:4, particularly in the neutral lipid fraction. In most of these studies, 18:4 was not well resolved from the [1-^{14}C]18:3(n-3) substrate peak in radio-GLC analyses, appearing as a shoulder that was not reliably quantitated. At most, labeled 18:4 accounted for <4% of the product. Only with unlabeled 20:3(n-6) or 20:3(n-3) was there evidence of increased accumulation of labeled 18:4 in phospholipid (twofold) and neutral lipid (four- to sixfold).

Effects of varying the concentration of fatty acids on [1-^{14}C]18:3(n-3) and [1-^{14}C]18:2(n-6) metabolism. Several unlabeled fatty acids were examined over a concentration range of 10–200 μM for effects on [1-^{14}C]18:3(n-3) metabolism. All unlabeled acids decreased product formation and esterification to phospholipid in a concentration dependent manner. The most inhibitory was the 22:6(n-3), producing >85% inhibition of total product formation at 200 μM. In contrast, 20:3(n-3) was least inhibitory over the concentration range tested. Unlabeled 20:4(n-6) gave a dramatic shift of total product to the neutral lipid fraction but altered total product formation by <15%. Other unlabeled acids all exhibited their primary effect on neutral lipid labeling at 40 μM or less. When individual labeled products in the phospholipids were examined (Fig. 3), a concentration-dependent decrease in labeled 20:5 was seen. The enhanced formation of labeled 22:5 seen with unlabeled 18:3(n-6) or 20:4(n-6) decreased at concentrations >40 μM.

In previous studies (35,36), the influence of a wide variety of fatty acids on the metabolism of [1-^{14}C]18:2(n-6) was tested, but in those studies 20:5(n-3) was not examined. In the present study, markedly different effects of varying concentrations of the two unlabeled fatty acids, 20:3(n-3) and 20:5(n-3), were observed when the metabolisms of [1-^{14}C]18:3(n-3) and [1-^{14}C]18:2(n-6) were compared (Fig. 4). Alteration of labeled 18:2(n-6) metabolism required >40 μM concentrations of these two unlabeled acids, whereas inhibition of [1-^{14}C]18:3(n-3)

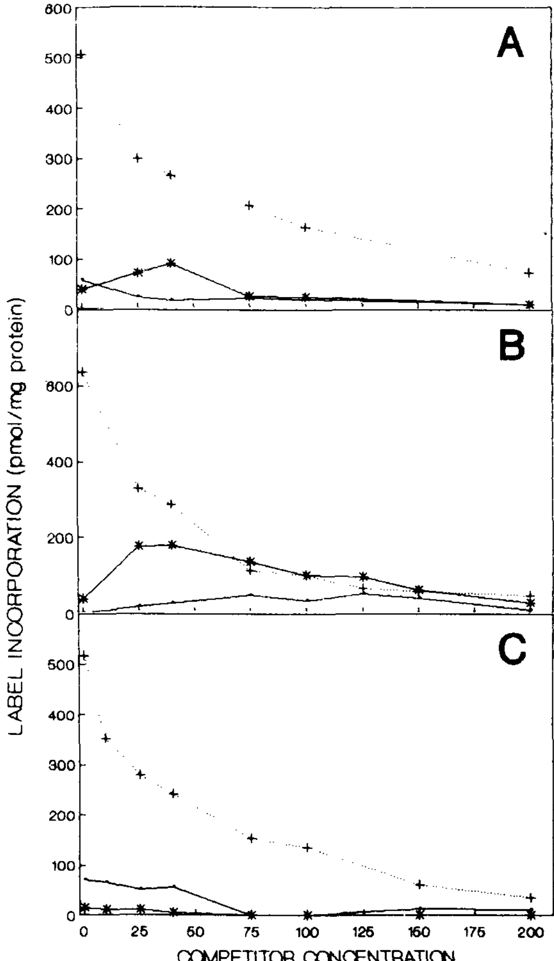

FIG. 3. **Influence of varying concentrations of fatty acids on the formation of individual products from [1-^{14}C]18:3(n-3) and their esterification to phospholipid. Glioma cells were incubated for 2 hr with 1 μCi [1-^{14}C]18:3(n-3) and various concentrations of unlabeled fatty acids. Lipid extracts were separated on silicic acid columns. Fatty acid methyl esters obtained from the neutral lipid and phospholipid fractions were analyzed by radio gas liquid chromatography. (A) Unlabeled 18:3(n-6) as competitor; (B) 20:4(n-6) as competitor; (C) 22:6(n-3) as competitor. Label in 20:4, —●—; label in 20:5, ···+···; label in 22:5, —*—.**

metabolism was observed even at the lowest concentration tested.

Product distribution in individual lipids and the influence of lysophosphatidylcholine. When distribution of products in individual lipids was examined (Fig. 5), most of the label was found in phosphatidylcholine; this was slightly reduced in the presence of unlabeled 20:4(n-6). Phosphatidylethanolamine also contained a large portion of the product, particularly 22:5. Triacylglycerol (panel E) contained similar amounts of each of the products, and esterification to triacylglycerol was increased in the presence of 40 μM 20:4(n-6). With 40 μM [^{3}H]lysophosphatidylcholine in the incubation, 9–13% of the [^{3}H]label from lysophosphatidylcholine was incorporated into the cell lipid extract; of this, >87% was in phosphatidylcholine. The presence of lysophosphatidylcholine had relatively little influence on the formation and esterification of products; there was a slight increase (6–16%) in label esterified to phosphatidylcholine, with corresponding slight decrease in label in phosphatidylethanolamine and triacylglycerol.

DISCUSSION

Complex interactions between fatty acids of the (n-3) and (n-6) polyunsaturated families have been shown in the desaturation, chain elongation and esterification reactions in our cultured glioma cells. Condensed summaries of the results of this and our previous studies (35,36) are presented in Table 1 (incorporation into phospholipid) and Table 2 (incorporation into neutral lipid). Previously we

TABLE 1

Effects of Fatty Acid Competitors: Total Phospholipid

Fatty acid competitor	18:2(n-6) Metabolism			18:3(n-3) Metabolism			
	Total	20:3	20:4	Total	20:4	20:5	22:5
Saturates and monoenes	—	—	—	—	—	—	—
18:2(n-6)	↑↑	—	↑↑	↓	↓↓	↓↓	↑↑
18:3(n-6)	↑↑	↑↑	↑	↓	↑	↓↓	↑↑
20:3(n-6)	↑↑	—	↑↑	↓	—	↓↓	↑↑
20:4(n-6)	↑↑	↓	↑↑	↓	↓↓	↓↓	↑↑
18:2(n-6)tt	↓↓	↓	↓↓	↓↓	↑	↓↓	↓↓
18:3(n-3)	↓	↓	↓	↓↓	↓↓	↓↓	↓↓
20:3(n-3)	—	—	—	↓	↑	↓	↓↓
20:5(n-3)	—	—	—	↓	↓↓	↓	—
22:6(n-3)	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓	↓↓
Control levels (pmol/mg protein)	260	150	110	765	38	664	52

Results are shown for 40 μM competitor fatty acid. Control values are for 4 hr incubations with 18:2(n-6) as substrate and for 2 hr incubation with 18:3(n-3). —, No change (<±10%); ↑, 1.1- to 1.5-fold increase; ↑↑, >1.5-fold increase; ↓, 10–30% decrease; ↓↓, 30–95% decrease.

TABLE 2

Effects of Fatty Acid Competitors: Total Neutral Lipid

Fatty acid competitor	18:2(n-6) Metabolism			18:3(n-3) Metabolism			
	Total	20:3	20:4	Total	20:4	20:5	22:5
Saturates and monoenes	—	—	—	—	—	—	—
18:2(n-6)	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑	↑↑
18:3(n-6)	↑↑	↑↑	—	↑↑	↑↑	↑↑	↑↑
20:3(n-6)	↑↑	↑↑	↓	↑↑	↑↑	↓↓	↑↑
20:4(n-6)	↑↑	↑↑	—	↑↑	↑↑	↑↑	↑↑
18:2(n-6)tt	↓↓	↓	↓↓	—	↑	↓↓	↓↓
18:3(n-3)	↓	—	↓	↑↑	↑↑	↑↑	↓
20:3(n-3)	↑	↑	↓	↑↑	↑↑	—	↑↑
20:5(n-3)	↑	↑	↓	↑↑	↑↑	↓	↑↑
22:6(n-3)	↓↓	↓↓	↓↓	↑↑	↑↑	—	↑
Control levels (pmol/mg protein)	26	14	12	32	13	9	7

Results are shown for 40 μM competitor fatty acid. Control values are for 4 hr incubations with 18:2(n-6) as substrate and for 2 hr incubation with 18:3(n-3). —, No change (<±10%); ↑, 1.1- to 1.5-fold increase; ↑↑, >1.5-fold increase; ↓, 10–30% decrease; ↓↓, 30–95% decrease.

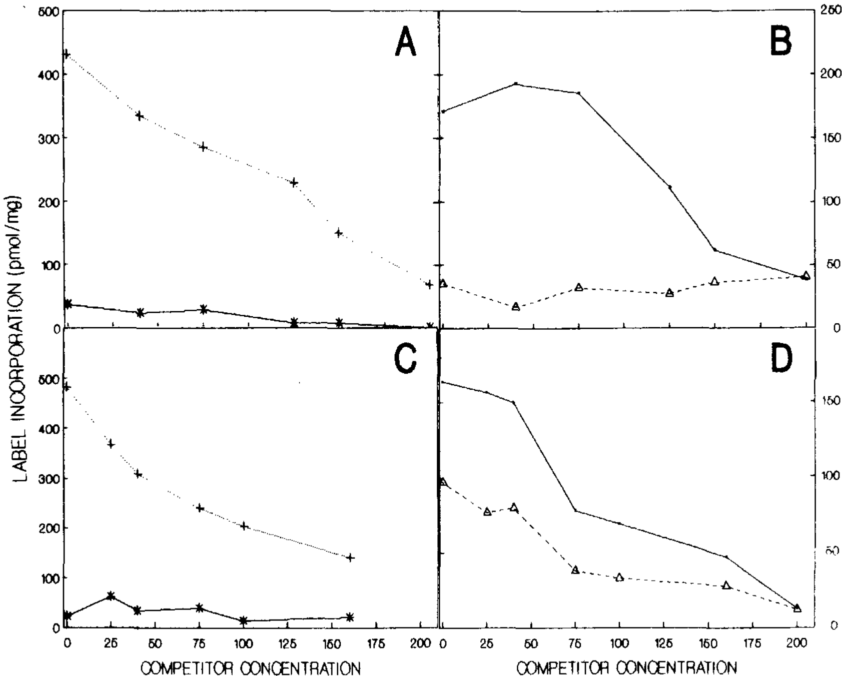

FIG. 4. Influence of varying concentrations of 20:3(n-3) or 20:5(n-3) on the metabolism of [1-^{14}C]18:3(n-3) or [1-^{14}C]18:2(n-6) in glioma cells. Cells were incubated with 1 μCi [1-^{14}C]18:3(n-3) (panels A and C) for 2 hr or with 1 μCi [1-^{14}C]18:2(n-6) (panels B and D) for 4 hr in the presence of varying concentrations of 20:3(n-3) (panels A and B) or 20:5(n-3) (panels C and D). Lipids were extracted, separated on silicic acid columns, transesterified and analyzed by radio gas liquid chromatography. Labeled products in the phospholipid fraction were 20:3, — —△— —; 20:4, —●—; 20:5, ····+····; 22:5, —*—.

investigated potential mechanisms behind the marked stimulation of 18:2(n-6) metabolism by (n-6) fatty acids (36). We concluded that only part of the interaction may relate to the relative availability of phospholipid and neutral lipid acceptors and competition for these acceptors by a mixture of fatty acid isomers. Triacylglycerol appears to serve as a preferred donor of acyl chains for further desaturation and chain elongation. However, we also concluded that there must also be a highly specific effect of certain individual fatty acid isomers on the functional desaturation-chain elongation-esterification complex; some fatty acids with similar specificities for esterification to phospholipid and neutral lipid [e.g., 20:3(n-6) and 20:3(n-3)] have markedly different effects on the desaturation and chain elongation of 18:2(n-6).

In this study, we concentrated on the metabolism of 18:3(n-3). The major product of desaturation and chain elongation of 18:3(n-3) in glioma cells was 20:5, which was almost exclusively esterified to phospholipids, even when the distribution of other products markedly shifted to triacylglycerol. On a quantitative basis, 20:5 showed the largest change in the presence of other fatty acids, although in some instances greater changes relative to control values were seen with the minor products. The (n-6) fatty acids had little effect on total product formation but appeared to be better competitors for a limited phospholipid pool, diverting products from phospholipid to triacylglycerol. There was an increased accumulation of 22:5 with an approximately corresponding decrease in 20:5. The 20:3(n-6) appeared to have somewhat special properties as a competitor acid in that it markedly lowered 20:5 accumulation and enhanced 20:3, 20:4 and 22:5 accumulation, the latter to levels almost equivalent to 20:5. Accordingly, even within families, fatty acids differing only by the number of double bonds had markedly different effects.

The effects of individual acids seemed even more specific to the (n-3) fatty acids. Primarily by isotope or substrate dilution, exogenous 18:3(n-3) reduced accumulation of products in phospholipid with some increase in esterification of 20:3, 20:4 and 20:5 to triacylglycerol. [This is in distinct contrast to the marked substrate stimulation seen with 18:2(n-6) metabolism, where addition of 40 μM unlabeled 18:2(n-6) gave a three- to sixfold stimulation; 35,36.] The 20:3(n-3) decreased product formation from 18:3(n-3) but caused some accumulation of 20:4 in both phospholipid and neutral lipid, unlike its distinctive lack of effect on 18:2(n-6) metabolism. The 20:5(n-3) slightly decreased all of the products of 18:3(n-3) metabolism except for 22:5.

The most highly unsaturated fatty acid that accumulates from 18:3(n-3) metabolism in most tissues (but not in our transformed glioma cells) is 22:6(n-3). When supplied in the culture medium, this fatty acid was inhibitory at all concentrations tested. This inhibition, seen in other studies (33), represents a dramatic example of end-product feedback in the case with 18:3(n-3) as substrate, but also represents general inhibition as seen with 18:2(n-6) as substrate. (Special precautions were taken to avoid oxidation of this fatty acid, and three different commercial preparations were tested with similar results.)

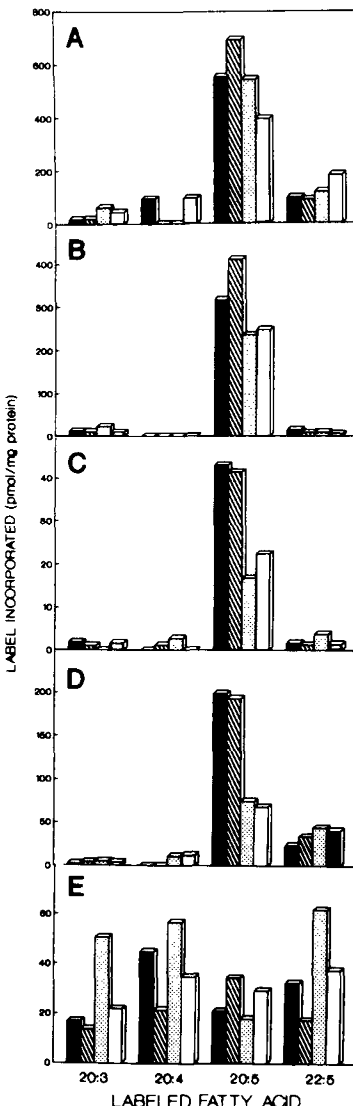

FIG. 5. Influence of lysophosphatidylcholine and 20:4(n-6) on the metabolism of [1-^{14}C]18:3(n-3) and distribution in various lipid fractions in glioma cells. Cells were incubated for 2 hr with 1 μCi [1-^{14}C]18:3(n-3) with the following additions to the medium: solid bar, control—no lysophosphatidylcholine or 20:4(n-6) added; hatched bar, 40 μM [^{3}H-methyl]lysophosphatidylcholine (2.5 μCi); dotted bar, 40 μM 20:4(n-6); open bar, 40 μM [^{3}H-methyl]lysophosphatidylcholine and 40 μM 20:4(n-6). Lysophosphatidylcholine and 20:4(n-6) were suspended in bovine serum albumin as described in Materials and Methods. Lipids were extracted and an aliquot was transesterified. The remainder was separated on thin layer chromatography; individual lipids were transesterified and methyl esters were analyzed on radio gas liquid chromatography. Labeled products are shown on the X-axis. (A) Label in total lipid; (B) label in phosphatidylcholine; (C) label in phosphatidylinositol; (D) label in phosphatidylethanolamine; (E) label in triacylglycerol.

Studies in experimental animals have indicated that the relative proportion of (n-6) and (n-3) fatty acids influences plasma and tissue lipid composition not only directly, by providing acyl chains for esterification to complex lipids, but also by altering the relative rates of conversion of fatty acids of the other family by desaturation and/or chain elongation. For example, an inverse relationship between 20:5(n-3) [but not 22:6(n-3)] and the ratio of 20:4(n-6) to 20:3(n-6) in plasma and liver lipids has been reported (23); the authors proposed that it is the 20:5(n-3) but not the 22:6(n-3) in fish oil that exerts an inhibitory effect on the conversion of 20:3(n-6) to 20:4(n-6). Such does not appear to be the case in our cells; 22:6(n-3) was strongly inhibitory and 20:5(n-3) had little effect on 18:2(n-6) conversion.

It is difficult to assign primary effects of competitive fatty acids specifically to an individual step in the desaturation-chain elongation-esterification sequence when a concerted action of these enzymes (35,36) may be involved. It appears that with 18:3(n-3) as substrate, an influence may occur at the Δ5 desaturation step resulting in a reduction in accumulation of 20:5, normally the major product, but enhanced chain elongation of 20:5 to 22:5 also contributes to this reduction. This contrasts with 18:2(n-6) metabolism, where overall product formation was enhanced as much as 10-fold (35) and Δ5 desaturation appeared to be stimulated. However, specific studies with [1-^{14}C]20:3(n-6) as substrate (35) did not directly support this; competitor acids had little effect compared to their major influences on the overall conversion of 18:2(n-6) to 20:4(n-6). On the other hand, accumulation of individual products seems to be greatly enhanced by the presence of exogenous precursors, suggesting that in some instances a particular step may be modulated. In the case of an end-product, such as 22:6(n-3), accumulation of each product or intermediate is reduced. Overall, the individual fatty acids seem to have greater influence than the availability of potential glyceride acceptors of the acyl chain products (such as lysophosphatidylcholine), although it may be that in the latter case the intracellular location of the appropriate form of the acyl chain acceptor may determine its effectiveness in altering 18:2(n-6) (36) or 18:3(n-3) metabolism.

In conclusion, our studies using radiolabeled substrates and unlabeled fatty acid competitors in intact cells have shown that the two families of (n-6) and (n-3) fatty acids interact differently to influence accumulation of different products or intermediates of desaturation and chain elongation, depending on the essential fatty acid precursor. Accordingly, the balance of these dietary fatty acids can markedly influence enzymes providing crucial membrane components and substrates for a variety of oxygenated, biologically active derivatives.

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Labeled Oxidation Products from [1-^{14}C], [U-^{14}C] and [16-^{14}C]-Palmitate in Hepatocytes and Mitochondria

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When [1-^{14}C], [U-^{14}C], and [16-^{14}C]palmitate were oxidized by isolated rat hepatocytes, there was a differential distribution of label as a percent of total oxidized products, such that $^{14}CO_2$ from [1-^{14}C] > [U-^{14}C] > [16-^{14}C]-palmitate and acid-soluble radioactivity from [16-^{14}C] > [U-^{14}C] > [1-^{14}C]palmitate. The oxidation of [2,3-^{14}C]succinate to $^{14}CO_2$ by isolated hepatocytes was only 9.1% of that from [1,4-^{14}C]succinate, demonstrating that the differences in distribution of labeled products are in part due to less $^{14}CO_2$ production from label in the even carbon positions entering the citric acid cycle. Apparent total ketone body production from [16-^{14}C]palmitate was markedly higher than [1-^{14}C] and [U-^{14}C]palmitate. In addition, the ^{14}C-acetone:$^{14}CO_2$ ratio derived from decarboxylation of labeled acetoacetate from [1-^{14}C]palmitate was less than 1 and positively correlated to the rate of fatty acid oxidation in hepatocytes. These findings indicate that the known preferential incorporation of the omega-C_2 unit of fatty acids into ^{14}C-ketone bodies also contributed to the differential distribution of labeled products and that this contribution was greatest at the lower rates of fatty acid oxidation. In isolated mitochondria, the distribution of label to $^{14}CO_2$ and acid-soluble radioactivity from [1-^{14}C], [U-^{14}C] and [16-^{14}C]palmitate was qualitatively similar to that seen with hepatocytes. The distribution of label from [1-^{14}C]acetylcarnitine to $^{14}CO_2$ and ^{14}C-ketone bodies by mitochondria was identical to that observed from [1-^{14}C]palmitate, indicating that the higher rates of $^{14}CO_2$ production from [1-^{14}C]palmitate cannot be explained by a preferential oxidation in the citric acid cycle of either extramitochondrial acetyl-CoA (generated in peroxisomes) or the carboxyl terminal of the fatty acid. As shown by others in cell-free systems, we observed that the total oxidation of [16-^{14}C]palmitate by hepatocytes and mitochondria was significantly less than [1-^{14}C] and [U-^{14}C]palmitate, suggesting either incomplete mitochondrial β-oxidation or incomplete degradation of peroxisomal oxidation products. The data indicate that this incomplete oxidation does not, however, contribute to the differential distribution of label to oxidized products.

Lipids 22, 620-626 (1987).

Fatty acids labeled in different carbon positions are commonly used to study the distribution of labeled oxidation products. In a preliminary study (1), we observed major differences in the distribution of label into CO_2 and acid-soluble products when [1-^{14}C], [U-^{14}C] and [16-^{14}C]palmitate were incubated with isolated hepatocytes and mitochondria from rat liver. From these experiments, it was clear that different conclusions regarding the rate of fatty acid utilization might be reached, depending on the position of label in the fatty acid. This is particularly relevant to studies quantitating the rate of fatty acid oxidation through the accumulation of a single product, such as $^{14}CO_2$ or ^{14}C-ketone bodies. To understand our preliminary observations, we conducted a more detailed study of this phenomenon.

Observations similar to those made in our preliminary study have been made by others in whole homogenates, postnuclear and mitochondrial fractions of various tissues (2) and isolated rat hepatocytes (3,4). In 1954, Brown et al. (5), using palmitic acid labeled in various carbon positions, indicated that an odd carbon of palmitic acid produced more $^{14}CO_2$ than did an even carbon in rat liver slices. Other laboratories (3,4,6,7) confirmed these findings. Additionally, Weinman et al. (8) found that more $^{14}CO_2$ is generated by the citric acid cycle from [1-^{14}C]acetyl-CoA than from [2-^{14}C]acetyl-CoA, which would be derived from odd and even labeled fatty acid carbons, respectively.

An alternate pathway in the liver for fatty acid-derived acetyl-CoA is toward acetoacetate synthesis. In 1944, Weinhouse et al. (9) reported that the incubation of [1-^{14}C]octanoate with rat liver slices produced acetoacetate labeled equally in the carboxyl and carbonyl carbons. Crandall et al. (10) showed that octanoate labeled in different carbon atoms gives rise to two different types of C_2 units. It was proposed that all C_2 units derived from a fatty acid chain, except the terminal C_2 units from the methyl end, are metabolically equivalent (11). Subsequent studies (12-17) demonstrated that the omega-terminal C_2 unit of fatty acids labeled in odd and even numbered carbons and of different chain lengths are preferentially incorporated into ketone bodies.

In the present investigation, we have looked in a single combined study at previously, but separately, studied phenomena to determine the contribution of each to the unequal distribution of labeled oxidation products from differentially labeled fatty acids. Additionally, these experiments bring to focus potential problems in the proper interpretation of data from labeled fatty acids in studies of fatty acid oxidation. These are problems that can lead to erroneous conclusions if the limitations of the methodology are not understood. Our studies also allow us to comment on the suitability of differentially labeled fatty acids for the study of fatty acid oxidation.

MATERIALS AND METHODS

Materials. ATP, NAD$^+$, coenzyme A, 3-hydroxybutyrate dehydrogenase, carnitine acetyl-transferase, malic dehydrogenase, DTT (dithiothreitol), MES [2-(N-morpholino) ethanesulfonic acid], palmitic acid and Tris (trizma base) were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C], [U-^{14}C], [16-^{14}C]palmitate, [1-^{14}C]octanoate and [1,4-^{14}C], [2,3-^{14}C]succinic acid were purchased from New England Nuclear (Boston, MA). [1-^{14}C]acetyl-CoA was purchased from ICN Biochemicals (Irvine, CA). Bovine albumin (CRG-7, fatty acid-free) was from

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Armour Pharmaceutical Co. (Tarrytown, NY). L-carnitine was generously provided by Dr. Yuzo Kawashima of the Otsuka Pharmaceutical Factory, Inc. (Tokushima, Japan). Hydrofluor was purchased from National Diagnostics (Somerville, NJ). All other chemicals were reagent grade.

Animals. Male Wistar rats (Charles River, Wilmington, MA) weighing 200–250 g at the time of use were maintained on Purina Rat Chow with free access to food and water. All animals were housed in rooms with controlled temperature and a 12 hr/12 hr, light/dark cycle (light from 7 a.m. to 7 p.m.).

Isolation and incubations of hepatocytes. Hepatocytes were prepared by the method of Berry and Friend (18), modified as described by others (19,20). To determine whether hepatocytes satisfied the criteria defined by Krebs et al. (20) for intact, metabolically active hepatocytes, ATP was measured (21) on the cell preparations. Hepatocytes (80–100 mg wet weight) were preincubated in a total volume of 1.5 ml for 45 min at 37 C in Krebs-Henseleit medium (22) containing 13.3 mM glucose, 13.3 mM lactate, 2.7 mM pyruvate and 1.3 mM L-carnitine under an atmosphere of 95% O_2/5% CO_2. Following preincubation, 0.5 ml of ^{14}C-palmitate/albumin complex in Krebs-Henseleit medium was added to a final concentration of 1.5 mM/4% (except where noted), bringing the final volume to 2.0 ml. Incubations after the addition of fatty acid were carried out for 15 min. Previous studies have indicated that this incubation system yields linear rates of fatty acid oxidation over the entire 15-min incubation period (23).

Isolation and incubation of liver mitochondria. Rat liver mitochondria were isolated from ad libitum-fed rats as previously described (24,25). The final pellet was resuspended in 0.25 M sucrose, 3 mM Tris/HCl (pH 7.4 at 4 C) to provide a concentration of ca. 20 mg of mitochondrial protein/ml. All mitochondrial preparations had a respiratory control ratio (26) above 5 with the substrates 5 mM glutamate plus 5 mM malate.

Mitochondria (ca. 5 mg of protein) were incubated at 37 C in a system of 94 mM sucrose, 60 mM KCl, 10 mM potassium phosphate, 5 mM Mg Cl_2, 3 mM ATP, 20 μM CoA, 1 mM DTT, 0.4 mM L-carnitine and 0.5 mM palmitate/1.5% albumin in a total volume of 2 ml at a pH of 7.1–7.2. Incubations were carried out for 15 min, during which time rates of fatty acid oxidation were linear (data not shown). Protein was measured according to Lowry et al. (27) with modifications (28).

Measurement of $^{14}CO_2$. Hepatocytes and mitochondria were incubated with the respective labeled substrates in sealed flasks that were fitted with centerwells containing folded filter paper. After incubations were terminated by the injection of 0.2 ml of cold 62.5% citric acid, 0.2 ml of 20% KOH was injected into the center wells (29). The sealed flasks were incubated at 37 C for an additional 1 hr to trap $^{14}CO_2$. The center wells were then removed and placed into scintillation vials containing 1 ml of H_2O and 10 ml of hydrofluor and were counted for radioactivity in a liquid scintillation counter.

Measurement of acid-soluble radioactivity and ^{14}C-ketone bodies. For the measurement of acid-soluble radioactivity and ^{14}C-ketone bodies, the reactions were terminated with 0.3 ml of cold 60% perchloric acid ($HClO_4$). Acid-soluble radioactivity was determined on hexane-washed perchloric acid extracts. After 0.5 ml of the $HClO_4$ extracts was removed for ^{14}C-ketone body determination, the remaining extracts were washed with 5 ml of hexane by vigorous shaking in tightly capped culture tubes and were centrifuged at 500 × g for 10 min. The top hexane layer was carefully aspirated and discarded. The extract was then washed for a second time following the same procedure. Hexane extraction removes any remaining ^{14}C-fatty acids. Aliquots of the washed extract were taken and counted for radioactivity. Total oxidized products refers to the sum of $^{14}CO_2$ plus the acid-soluble radioactivity.

The determination of ^{14}C radioactivity in ketone bodies was performed on neutralized (pH 6.0-6.5 with 4 M KOH, 0.25 M MES) perchloric acid extracts exactly by the method of Mewes et al. (30). This method allows for direct determination of radioactivity in the acetone and carboxyl moieties of acetoacetate after converting 3-hydroxybutyrate to acetoacetate in the presence of NAD^+ and 3-hydroxybutyrate dehydrogenase. After terminating the reaction with 5 N H_2SO_4, the acetoacetate formed was decarboxylated in sealed reaction vessels by heating to 100 C. The released ^{14}C-acetone and $^{14}CO_2$ were trapped in 1.5 ml of hydrazine lactate solution [10 ml of 90% DL-lactic acid + 4 ml of 100% hydrazine hydrate, pH 5–5.5 (30)] and 0.2 ml of 20% KOH, respectively, and counted for radioactivity. Statistical significance was determined using Student's t-test for unpaired data.

RESULTS AND DISCUSSION

The data in Table 1 demonstrate the differential distribution of label between $^{14}CO_2$ and acid-soluble radioactivity when isolated hepatocytes were incubated with [1-^{14}C], [U-^{14}C] and [16-^{14}C]palmitate. When expressed as a percent of total oxidized products, $^{14}CO_2$ production from [1-^{14}C] > [U-^{14}C] > [16-^{14}C]palmitate and the acid-soluble radioactivity from [16-^{14}C] > [U-^{14}C] > [1-^{14}C]palmitate. The total recoveries of label in products ($^{14}CO_2$ + acid soluble radioactivity) from [1-^{14}C] and [U-^{14}C]palmitate were not significantly different, indicating that both were oxidized at the same rate, but with different distribution of labeled carbon. Since ^{14}C-ketone body production from these fatty acids was also the same, these data suggest the difference was in the oxidation of labeled carbon entering the citric acid cycle. An experiment was conducted to determine if the difference in label distribution to $^{14}CO_2$ was the result of a lag in the rate of appearance of label in the even vs the odd carbon positions. We noted that the rate of appearance of $^{14}CO_2$ from [1-^{14}C] and [U-^{14}C]palmitate was linear throughout a 10-min incubation period, and the difference between the two labels (as observed in Table 1) was apparent from the earliest time point (0.5 min) to the end of the incubation period (data not shown). Therefore there is no lag in the appearance of label in the even vs the odd carbon positions, but these data do indicate that the system was in isotopic steady state. A single time point was measured in all subsequent experiments.

The apparent rate of total oxidation of [16-^{14}C]palmitate was significantly less than [1-^{14}C] and [U-^{14}C]palmitate, suggesting incompletely oxidation of the fatty acids. This is in contrast to earlier reports (3,4) where the total

TABLE 1

Distribution of Labeled Products from the Oxidation of [1-^{14}C], [U-^{14}C] and [16-^{14}C]Palmitate by Isolated Rat Hepatocytes

	Apparent rates of product production (nmol of ^{14}C-fatty acid converted to products/min/g cells)				
				^{14}C-Ketone bodies	
Substrate	$^{14}CO_2$	Acid-soluble radioactivity	Total oxidized products	Total production	^{14}C-Acetone:$^{14}CO_2$
1.5 mM [1-^{14}C]palmitate/4% albumin	38.2 ± 0.4 (15.6 ± 0.9%)	207.3 ± 13.6 (84.4 ± 0.9%)	245.5 ± 13.6	118.9 ± 11.7 (48.4 ± 6.3%)	0.81 ± 0.08
1.5 mM [U-^{14}C]palmitate/4% albumin	15.6 ± 0.7 (6.7 ± 0.5%)	217.3 ± 15.5 (93.3 ± 0.5%)	232.9 ± 15.5[a]	115.3 ± 0.1[a] (49.2 ± 4.4%)	2.91 ± 0.12
1.5 mM [16-^{14}C]palmitate/4% albumin	1.4 ± 0.3 (0.7 ± 0.2%)	199.9 ± 13.0 (99.3 ± 0.2%)	201.2 ± 12.8[b,c]	154.7 ± 21.0[b,c] (76.9 ± 9.7%)	—

Values are the mean of at least three separate hepatocyte preparations ± standard deviation. Values in parentheses represent the percentage of total oxidized products. Following a 45-min preincubation, heptocytes were incubated for 15 min at 37 C with the indicated fatty acid substrate as described in Materials and Methods.

[a]Not significantly different compared to [1-^{14}C]palmitate.

[b]Significantly different compared to [1-^{14}C]palmitate; $p < 0.01$.

[c]Significantly different compared to [U-^{14}C]palmitate; $p < 0.05$.

oxidation rates between [16-^{14}C] and [1-^{14}C]palmitate in isolated hepatocytes were found to be the same. A possible explanation for this discrepancy is that our incubation conditions and cell concentration differed from those used in these earlier studies.

Apparent total ketone body production from [16-^{14}C]-palmitate was much higher than that of [1-^{14}C] and [U-^{14}C]palmitate. These findings are consistent with earlier reports (12-17) that the last two carbons of fatty acids do not enter the acetyl-CoA pool but rather go preferentially to ketone body production. If the last two carbons of the fatty acid were preferentially incorporated into ketone bodies, these carbons would reside in the terminal methyl and carbonyl carbons of acetoacetate. That this occurs is supported by our observation that, in the presence of [1-^{14}C]palmitate, the ^{14}C-acetone:$^{14}CO_2$ ratio (the ratio of ^{14}C-acetone:$^{14}CO_2$ in the case of [1-^{14}C]-palmitate represents the distribution of label in the carbonyl vs the carboxyl carbons of acetoacetate) was less than 1 (0.81), suggesting a dilution of label in the carbonyl carbon of acetoacetate. The fact that the ^{14}C-acetone:$^{14}CO_2$ ratio with [U-^{14}C]palmitate was approximately equal to the theoretical ratio of 3 provides support for the validity of this method in the present study (see also Table 4).

The data of Table 1 also emphasize an important consideration when using fatty acids labeled in different carbon positions to measure rates of fatty acid oxidation. The calculation of true product production rates from labeled fatty acids requires that three basic assumptions be met: (i) that the labeled fatty acids (with the exception of those uniformly labeled) be completely oxidized; (ii) that all the labeled carbon undergoing β-oxidation enter a common acetyl-CoA pool before utilization (again, not a requirement of [U-^{14}C]palmitate), and (iii) that all labeled carbon, regardless of position, demonstrate the same distribution in the various products. Our data clearly show that these assumptions are not completely satisfied for any of the fatty acids used in these studies. Therefore, this must be considered when measuring rates of production of the individual products. In reference to this consideration, we have termed product production rates as "apparent" (see Tables 1 and 4). Since the data with [16-^{14}C]palmitate indicate that the oxidation is not complete, we have chosen to compare the distribution of labeled carbon between the differentially labeled fatty acids as a percent of total oxidized products (parentheses in Tables 1 and 4).

The experiments of Table 2 were conducted to determine whether the distribution of labeled products as shown in Table 1 could be explained, in part, by the position of the label in the fatty acid. We observed that $^{14}CO_2$ production from [2,3-^{14}C]succinate was only 9.1% of that from [1,4-^{14}C]succinate, a value that compares very favorably with that reported by Bessman et al. (31). It is known that several turns through the citric acid cycle would be required to convert all the label in [2,3-^{14}C]succinate to $^{14}CO_2$, compared to one turn for [1,4-^{14}C]succinate. In addition, Zaidise et al. (32) showed that about 95% of succinate utilized by hepatocytes does not complete one full turn through the citric acid cycle. Since [2,3-^{14}C]succinate would be derived from labeled carbon in the even position of the fatty acid ([U-^{14}C] and [16-^{14}C]palmitate) and [1,4-^{14}C]succinate from labeled carbon in the odd position ([1-^{14}C] and [U-^{14}C]palmitate), these data support the conclusion that the differences in the label distribution between [1-^{14}C], [U-^{14}C] and [16-^{14}C]palmitate are at least partially due to less $^{14}CO_2$ production from label in the even carbon positions of the fatty acid, with consequently more label remaining in the acid-soluble fraction.

However, if this were the only explanation, then based on the labeled succinate data one would expect $^{14}CO_2$ from [U-^{14}C] and [16-^{14}C]palmitate to be ca. 55% (50% + 0.5 × 9.1%) and 9.1%, respectively, of that from [1-^{14}C]palmitate (expressing $^{14}CO_2$ as a percentage of total oxidized products). $^{14}CO_2$ production from these fatty acids was less than expected (42.9% and 4.4%, respectively), indicating that other factors are involved in determining

TABLE 2

Comparison of $^{14}CO_2$ Production from Labeled Fatty Acid and Succinate by Isolated Hepatocytes

Substrate	$^{14}CO_2$ as percentage of total oxidized products[b]	DPM recovered as $^{14}CO_2$	$^{14}CO_2$ production as % of $^{14}CO_2$[a]	
			From [1-^{14}C]palmitate	From [1,4-^{14}C]succinate
1.5 mM [1-^{14}C]palmitate/4% albumin	15.6 ± 0.7%	—	100	—
1.5 mM [U-^{14}C]palmitate/4% albumin	6.7 ± 0.5%	—	42.9	—
1.5 mM [16-^{14}C]palmitate/4% albumin	0.7 ± 0.2%	—	4.4	—
1.5 mM palmitate/4% albumin and trace [1,4-^{14}C]succinate[c]	—	90,167 ± 8,798	—	100
1.5 mM palmitate/4% albumin and trace [2,3-^{14}C]succinate[c]	—	8,214 ± 298	—	9.1

Values from succinate experiments are the average of two experiments ± the range. Each experiment was conducted with triplicate incubations utilizing the same hepatocyte preparations of Table 1. Following a 45-min preincubation, hepatocytes were incubated for 15 min at 37 C with the indicated fatty acid and succinate substrate as described in Materials and Methods.

[a]Calculations were based upon the values for $^{14}CO_2$ from [1-^{14}C]palmitate and [1,4-^{14}C]succinate of columns one and two, respectively.

[b]Data taken from Table 1.

[c]Specific activities of [1,4-^{14}C] and [2,3-^{14}C]succinate were identical.

the distribution of labeled products between $^{14}CO_2$ and the acid-soluble pool. Taken together with the data of Table 1, it seems likely that the preferential use of the omega-terminal C_2 unit for ketone body synthesis provides an additional explanation for the differential distribution of labeled products.

Huth et al. (33) demonstrated that the [^{14}C]carbonyl/[^{14}C]carboxyl ratio of acetoacetate from [1-^{14}C] fatty acids was highest (approaching 1.0) in animals with metabolic states characterized by high rates of ketone body production. They indicated that changes in this ratio were positively correlated to changes in the activity of acetoacetyl-CoA thiolase in the reverse reaction. In other words, under metabolic states yielding low rates of ketone body production, the activity of acetoacetyl-CoA thiolase would be low and unable to equilibrate acetoacetyl-CoA (generated in β-oxidation from the last four carbons of the fatty acid) with the acetyl-CoA pool, and thus there would be direct use of the omega-terminal C_4 unit of the fatty acid for acetoacetate synthesis. With [1-^{14}C]palmitate, this would result in the unlabeled omega-terminal C_2 unit being incorporated into the carbonyl carbon of acetoacetate without first entering the common acetyl-CoA pool, causing a lower ^{14}C-acetone:$^{14}CO_2$ ratio.

To determine if this ratio would vary with respect to rates of fatty acid oxidation in isolated hepatocytes from animals in a single metabolic state, but incubated under different conditions (high fatty acid concentration ± glucagon and low fatty acid concentration), the experiments reported in Table 3 were conducted. In agreement with the observations of Huth et al. (33), these data show that the rate of fatty acid oxidation in hepatocytes is positively correlated with the ^{14}C-acetone:$^{14}CO_2$ ratio of acetoacetate. Even though this relationship is not statistically significant with the small sample size, there is clearly a trend for a decreasing ratio with a decrease in oxidation rate. These data provide additional evidence that the ^{14}C-acetone:$^{14}CO_2$ ratio reflects the degree of preferential incorporation of the omega-terminal C_2 unit of the fatty acid into ketone bodies.

The data in Table 4 show the distribution of labeled products from the oxidation of [1-^{14}C], [U-^{14}C] and [16-^{14}C]-palmitate in isolated rat liver mitochondria. The $^{14}CO_2$ from ^{14}C-palmitate was much less, as a percentage of the total oxidized products, than that seen in isolated hepatocytes (Table 1), indicating that acetyl-CoA generated from fatty acid oxidation in the absence of other substrates is directed principally toward ketone body synthesis. However, as seen with hepatocytes (Table 1), $^{14}CO_2$ from [1-^{14}C]palmitate is greater than that from [U-^{14}C] and [16-^{14}C]palmitate. Unlike hepatocytes, ^{14}C-ketone body synthesis, as a percent of total oxidized products, was not greater with [16-^{14}C]palmitate. This could be explained by the fact that the majority of label in these experiments is directed to ketone body synthesis, whether from the acetyl-CoA pool or the terminal C_2 unit of the fatty acid. The ^{14}C-acetone:$^{14}CO_2$ ratio of 0.79 for [1-^{14}C]palmitate does, however, support preferential use of the omega-terminal C_2 unit.

In isolated mitochondria (Table 4), both the total oxidation of [U-^{14}C]palmitate and [16-^{14}C]palmitate were significantly less than that of [1-^{14}C]palmitate. The data indicate that 21% of the fatty acid carbon entering β-oxidation was not completely oxidized to CO_2 or acid-soluble fragments {1 − (total oxidized products from [U-^{14}C]palmitate/total oxidized products from [1-^{14}C]-palmitate)}. Additionally, it can be calculated that 45% of the fatty acids entering β-oxidation were incompletely oxidized down to acid-insoluble fragments containing carbon 16 {1 − (total oxidized products from [16-^{14}C]palmitate/total oxidized products from [1-^{14}C]palmitate)}. Considering these two points together, it can be stated that at the end of the incubation, 21% of the fatty acid carbon starting β-oxidation remained in acid-insoluble fragments containing 45% of the starting carbon 16. The average length of these acid insoluble fragments can be calculated from the relationship, $0.21 = 0.45x$, where x is the average fractional length of the fragment, or ca. 0.5. Therefore, for a 16 carbon fatty acid, the average fragment length would be 8.

TABLE 3

Influence of the Rate of Fatty Acid Oxidation on the Ketone Body Acetone:CO_2 Ratio in Isolated Hepatocytes

Substrate	nmol [1-^{14}C]Palmitate converted to total oxidized products/min/g cells	Ketone body ^{14}C-acetone:$^{14}CO_2$ ratio[a]
1.5 mM [1-^{14}C]Palmitate/[b] 4% albumin + 2 μM glucagon	292.5 ± 51.0	0.92 ± 0.05
1.5 mM [1-^{14}C]Palmitate/[c] 4% albumin	245.5 ± 13.6	0.81 ± 0.08
0.5 mM [1-^{14}C]Palmitate/[d] 4% albumin	50.7 ± 8.8	0.74 ± 0.11

Values are expressed as the mean of at least three separate hepatocyte preparations ± standard deviation.

[a]A correlation coefficient of 0.889 was determined by linear regression analysis of the data, indicating the influence of the rate of total oxidation on the ketone body ^{14}C-acetone:$^{14}CO_2$ ratio.

[b]Following a 45-min preincubation in the presence of 1 μM glucagon and 1.33 mM L-carnitine, hepatocytes were incubated for 15 min at 37 C with 1.5 mM [1-^{14}C]palmitate plus an additional 1 μM glucagon.

[c]Data taken from Table 1.

[d]Following a 45-min preincubation in the presence of glucose, lactate, pyruvate, and carnitine, as described in Materials and Methods, hepatocytes were incubated for 15 min at 37 C with 0.5 mM [1-^{14}C]palmitate.

TABLE 4

Distribution of Labeled Products from the Oxidation of [1-^{14}C], [U-^{14}C] and [16-^{14}C]Palmitate in Isolated Rat Liver Mitochondria

	Apparent rates of product production (nmol converted to products/min/mg mitochondrial protein)				
				^{14}C-Ketone bodies	
Substrate	$^{14}CO_2$	Acid-soluble radioactivity	Total oxidized products	Total production	^{14}C-Acetone:$^{14}CO_2$
0.5 mM [1-^{14}C]Palmitate/1.5% albumin	0.07 ± 0.01 (1.3 ± 0.0%)	5.38 ± 0.41 (98.8 ± 0.1%)	5.45 ± 0.41	3.45 ± 0.28 (63.4 ± 5.6%)	0.79 ± 0.10
0.5 mM [U-^{14}C]Palmitate/1.5% albumin	0.03 ± 0.01 (0.7 ± 0.1%)	4.30 ± 0.34 (99.4 ± 0.1%)	4.33 ± 0.34[a]	2.86 ± 0.49 (66.0 ± 6.0%)[b]	3.06 ± 0.49
0.5 mM [16-^{14}C]Palmitate/1.5% albumin	0.01 ± 0.00 (0.3 ± 0.1%)	2.97 ± 0.42 (99.7 ± 0.0%)	2.98 ± 0.42[c,d]	1.86 ± 0.23 (62.4 ± 4.7%)[b]	—

Values are the mean of three separate mitochondrial isolations ± standard deviation. Values in parentheses represent the percent of total oxidized products. Mitochondria were incubated for 15 min at 37 C with the indicated fatty acid substrate as described in Materials and Methods.

[a]Significantly different compared to [1-^{14}C]palmitate; $p < 0.05$.

[b]Not significantly different compared to [1-^{14}C]palmitate.

[c]Significantly different compared to [1-^{14}C]palmitate; $p < 0.01$.

[d]Significantly different compared to [U-^{14}C]palmitate; $p < 0.05$.

It is interesting to note that octanoic acid and its CoA and carnitine esters have been reported to be only partially acid-soluble (34). We have found that hexane washing of perchloric acid extracts, under our conditions for preparing the acid soluble fraction, resulted in a loss of ca. 90% of labeled octanoate (data not shown). Thus, fatty acid fragments of 8 or more carbons would not be detected in the acid-soluble fraction. Since peroxisomal β-oxidation would specifically generate 8 carbon fragments (acyl moieties of 8 or fewer carbon atoms are not oxidized by peroxisomes [35]), our data is consistent with the suggestion that incomplete oxidation of palmitate observed in our studies was caused, at least to some extent, by an inadequate mitochondrial degradation of peroxisomal oxidation products. Veerkamp has made similar conclusions based upon work in cell-free systems (2). As we observed in Tables 1 and 4, one might expect from the above discussion that the differences between total oxidation of [1-^{14}C], [U-^{14}C] and [16-^{14}C]palmitate would be greater in isolated mitochondria than in hepatocytes, since the ability to transfer peroxisomal oxidation products to mitochondria should be diminished in the mitochondrial system. However, from our data, it cannot be ruled out that some, if not all, of the differences between total oxidation of differentially labeled palmitate were due to incomplete mitochondiral β-oxidation.

The data in Table 5 show the distribution of labeled products from the oxidation of [1-^{14}C]acetylcarnitine and

TABLE 5

Distribution of Labeled Products from the Oxidation of [1-^{14}C]Acetylcarnitine and [1-^{14}C]Palmitate in Isolated Rat Liver Mitochondria

Substrate	DPMs recovered as products		
	$^{14}CO_2$	^{14}C-Ketone bodies	$^{14}CO_2$:^{14}C-Ketone bodies
0.5 mM Palmitate/1.5% albumin and trace [1-^{14}C]acetyl-CoA plus 5.3 units of carnitine acetyltransferase [EC 2.3.1.7]	440 ± 13	16,404 ± 6,049	0.027 ± 0.012
0.5 mM [1-^{14}C]Palmitate/1.5% albumin plus 5.3 units of carnitine acetyltransferase	1,010 ± 6	43,642 ± 4,087	0.023 ± 0.002

Values are the mean of three replicates ± standard deviation from a representative experiment. Mitochondria were incubated for 15 min at 37 C with the indicated substrates. [1-^{14}C]Acetylcarnitine was produced during the incubation by the addition of [1-^{14}C]acetyl-CoA and carnitine acetyltransferase. 0.25 μCi of [1-^{14}C]acetyl-CoA was added.

[1-^{14}C]palmitate in isolated rat liver mitochondria. By measuring label appearing in $^{14}CO_2$ and ^{14}C-ketone bodies, we were able to determine whether acetyl-CoA generated extramitochondrially by peroxisomal oxidation and transported into the mitochondria enters the common acetyl-CoA pool or is preferentially directed toward the citric acid cycle. The latter possibility could offer an additional explanation for the higher rates of $^{14}CO_2$ production from [1-^{14}C] relative to [U-^{14}C] and [16-^{14}C]palmitate and from [U-^{14}C] relative to [16-^{14}C]palmitate. The data show, however, that both intra- and extramitochondrially generated acetyl-CoA are used equivalently, as indicated by the $^{14}CO_2$:^{14}C-ketone body ratios. This is in support of a single common acetyl-CoA pool. Even though the data of Table 4 suggest some contribution of peroxisomal oxidation to the overall rate, this does not appear to be involved in explaining the differential distribution of labeled oxidation products. These data also argue against the preferential use of acetyl units from the carboxyl terminal of [1-^{14}C]palmitate in the citric acid cycle.

CONCLUSIONS

The differential distribution of labeled products between [1-^{14}C], [U-^{14}C] and [16-^{14}C]palmitate in isolated rat hepatocytes and liver mitochondria can be explained (i) for the most part by the difference in $^{14}CO_2$ production from label in the odd or even carbon positions of fatty acid-derived acetyl-CoA entering the citric acid cycle and (ii) by the preferential incorporation of the omega-terminal C_2 unit of fatty acids into ketone bodies. (iii) The data support a single common mitochondrial acetyl-CoA pool regardless of whether the acetyl units are produced intra- or extramitochondrially. Therefore, the data are not consistent with an involvement of peroxisomal oxidation in the differential distribution of labeled products.

From these experiments we are also able to comment on the use of differentially labeled fatty acids for the study of fatty acid oxidation: (i) The use of [U-^{14}C]fatty acids gives the most accurate measurement of both the overall rate and extent of β-oxidation, particularly in mitochondrial and other cell-free systems in which oxidation may not be complete. It also best reflects incorporation of carbon into ketone bodies. However, citric acid cycle involvement would be underestimated. (ii) Use of [1-^{14}C]palmitate in our hepatocyte system gave data quite similar to [U-^{14}C]palmitate and more closely reflects citric acid cycle activity. (However, since cycle intermediates are continuously removed in a cellular system, it is unlikely that $^{14}CO_2$ production ever quantitatively reflects flux through this pathway.) Therefore, from a practical point of view, based on expense and availability, [1-^{14}C]fatty acids would be a better choice for studies of fatty acid oxidation in cells. In cell-free systems, however, one must consider the fact that fatty acid oxidation is probably not complete. It is clear from our data and that of others (2) that, regardless of the system or tissue used, one should always measure both $^{14}CO_2$ and acid-soluble radioactivity to determine accurate rates of *total* fatty acid oxidation. (iii) The use of [16-^{14}C]palmitate, or other omega-terminal labeled fatty acids for measuring the rate of fatty acid oxidation, is inappropriate due to the incomplete oxidation of fatty acids, especially in cell-free systems. Its use would also grossly underestimate $^{14}CO_2$ production and overestimate ^{14}C-ketone body production. However, combined with data obtained from [1-^{14}C] and [U-^{14}C]fatty acids, useful information on the degree of complete oxidation may be obtained, as illustrated in discussions above.

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Cholesterol Autoxidation in Phospholipid Membrane Bilayers

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Lipid peroxidation in unilamellar liposomes of known cholesterol-phospholipid composition was monitored under conditions of autoxidation or as induced by a superoxide radical generating system, γ-irradiation or cumene hydroperoxide. Formation of cholesterol oxidation products was indexed to the level of lipid peroxidation. The major cholesterol oxidation products identified were 7-keto-cholesterol, isomeric cholesterol 5,6-epoxides, isomeric 7-hydroperoxides and isomeric 3,7-cholestane diols. Other commonly encountered products included 3,5-cholestadiene-7-one and cholestane-3β,5α,6β-triol. Superoxide-dependent peroxidation required iron and produced a gradual increase in 7-keto-cholesterol and cholesterol epoxides. Cholesterol oxidation was greatest in liposomes containing high proportions of unsaturated phospholipid to cholesterol (4:1 molar ratio), intermediate with low phospholipid to cholesterol ratios (2:1) and least in liposomes prepared with dipalmitoylphosphatidylcholine and cholesterol. This relationship held regardless of the oxidizing conditions used. Cumene hydroperoxide-dependent lipid peroxidation and/or more prolonged oxidations with other oxidizing systems yielded a variety of products where cholesterol-5β,6β-epoxide, 7-ketocholesterol and the 7-hydroperoxides were most consistently elevated. Oxyradical initiation of lipid peroxidation produced a pattern of cholesterol oxidation products distinguishable from the pattern derived by cumene hydroperoxide-dependent peroxidation. Our findings indicate that cholesterol autoxidation in biological membranes is modeled by the peroxide-induced oxidation of liposomes bearing unsaturated fatty acids and suggest that a number of cholesterol oxidation products are derived from peroxide-dependent propagation reactions occurring in biomembranes.
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The oxidation of cholesterol in biological systems has received considerable attention for many years. There is general agreement that cholesterol oxidation in cells can result from enzymatic processes, as well as from free radical-mediated membrane lipid peroxidation (1,2). The 7α-hydroxylation of cholesterol during bile acid synthesis (3) is an example of microsomal cytochrome P-450 catalyzed oxidation. In the presence of its cofactors NADPH and O_2, cytochrome P-450 monooxygenase catalyzes peroxidation of microsomal lipids (4–6). This lipid peroxidation is accompanied by cholesterol autoxidation with the formation of several products, including the isomeric cholest-5-ene-3β,7β- and cholest-5-ene-3β,7α-diols (3,7-diols) (4,5), with the latter contributing to the enzymatically derived cholest-5-ene-3β,7α-diol (3).

Free radical oxidation of cholesterol yields several products that are commonly encountered in tissues and microsomal preparations. The radical species responsible for cholesterol oxidation appear to derive from activated oxygen. Accordingly, specific radical generating systems, or defined dioxygen species, have been employed in cholesterol oxidation studies using membraneous and nonmembraneous preparations (7–12). Moreover, activated oxygen appears to be generated by cytochrome P-450 monoxygenase (13,14) with the induction of lipid peroxidation (14) and cholesterol oxidation (15).

A qualitative similarity is apparent among cholesterol oxidation products formed in several artificial systems as well as those isolated from peroxidized biomembranes. In many cases, the nature of oxidizing species remains ill defined, or in those instances where defined species were utilized, the matrix within which cholesterol oxidation took place differed from the organized state of lipids in biomembranes. A thorough review of cholesterol oxidation products formed in aqueous colloidal solutions vs products isolated from natural sources is presented by Smith (16).

Oxidation of cholesterol in model membranes has recently been described for iron-catalyzed (10,11) and hydrogen peroxide- or ultraviolet light-induced autoxidations (17). These studies employed long periods of imposed oxidation (up to 24 hr) and utilized phospholipids containing varying degrees of unsaturation. The model membranes were in the form of large multilamellar vesicles in two reports (10,11) and small unilamellar vesicles in the other (17). In the former studies the membrane arrangement likely permitted direct access of oxidants generated in the aqueous phase to only a small proportion of lipids. The later study was concerned primarily with the stability of cholesterol and the effect of antioxidants, whereas little attention was given to product formation.

In this report the identities of the major detectable oxidation products of cholesterol in unilamellar vesicles (liposomes) are described. In addition, specific free radical generating systems have been used to induce lipid peroxidation with subsequent examination of cholesterol oxidation products. These liposomes afford a reasonably exact formulation of lipid composition permitting a measure of control over the mode and extent of peroxidation, conditions that are not readily achieved using membranes from cells.

MATERIALS AND METHODS

Chemicals and reagents. Bovine liver phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were purchased from Sigma (St. Louis, MO), and the solvents in which they were delivered were evaporated under a stream of argon. These lipids were then stored in methylene chloride at a concentration 5 mg/ml under argon at −80 C.

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; TBAR, thiobarbituric acid-reacting products; CuOOH, cumene hydroperoxide; HX, hypoxanthine; ADP, adenosine diphosphate; XO, xanthine oxidase; SOD, superoxide dismutase; TLC, thin layer chromatography; 7-keto, cholest-5-ene-3β-ol-7-one; 3,5-diene, 3,5-cholestadiene-7-one; α-CE, cholestan-5α,6α-epoxy-3β-ol; CT, 5α-6β-triol; 7-OOH, 3β-hydroxycholest-5-ene-7α-hydroperoxide; β-CE, cholestan-5β,6β-epoxy-3β-ol; HPLC, high pressure liquid chromatography; DPPC, dipalmitoylphosphatidylcholine; PI, peroxidizability index.

Various batches of these phospholipids were assayed for their content of thiobarbituric acid–reacting products (TBAR), and only samples containing no TBAR were used to prepare liposomes. Cumene hydroperoxide (CuOOH) and hypoxanthine (HX) were obtained from Aldrich (Milwaukee, WI); and cholesterol, adenosine diphosphate (ADP), xanthine oxidase (XO), bovine erythrocyte superoxide dismutase (SOD) and hematin were purchased from Sigma. 4[^{14}C]-Cholesterol was obtained from Amersham (Arlington Heights, IL) and repurified on a monthly basis by thin layer chromatography (TLC) as previously reported (18) to remove cholesterol oxidation products. A similar procedure was used to purify unlabeled cholesterol obtained from Sigma. The absence of cholesterol oxidation products was confirmed by gas and thin layer chromatography (19). The levels of cholesterol oxidation products were typically below our limits of detection.

Authentic cholesterol oxidation products: 3,7-diols, cholest-5-ene-3β-ol-7-one (7-keto), 3,5-cholestadiene-7-one (3,5-diene), cholestan-5α,6α-epoxy-3β-ol (α-CE) and 25-hydroxycholesterol were obtained from Sterloids (Wilson, NH). α-CE was found to be contaminated with 1% and 3% cholestan-3β,5α,6β-triol (CT) and cholesterol, respectively, and was further purified as described previously (20). CT was purchased from Sigma and was chromatographically pure. 3β-hydroxy-5α-cholest-6-ene-5α-hydroperoxide and 3β-hydroxycholest-5-ene-7α-hydroperoxide (7-OOH) were provided by Dr. Leland Smith. Cholestan-5β,6β-epoxy-3β-ol (β-CE) was prepared by reaction of cholesterol with metachloroperoxybenzoic acid in ether, as described previously (20). This reaction produces ca. 75% and 25% of the α- and β-CE diastereomers, respectively, requiring purification of β-CE by high pressure liquid chromatography (HPLC) (20).

Liposome preparation. Three different phospholipid formulations were used for preparing liposomes. These formulations were based on the type or proportions of phospholipids and consisted of either pure dipalmitoylphosphatidylcholine (DPPC), bovine liver PC or a mixture of PC and bovine liver PE in a molar ratio of 4:1. The phospholipids were mixed with cholesterol as methylene chloride solutions in quantities sufficient to achieve final desired molar ratios of phospholipid to cholesterol of either 2:1 or 4:1. At this point, a total of 8 μCi (at 56 μCi/μmol) of radiolabeled cholesterol was added to the sample. For a typical experiment, a total of 35 mg of lipid was mixed, and the solvent was evaporated under a stream of argon. The resulting film was suspended by vigorous mixing for 1 min in 2.4 ml of cold argon-saturated 10 mM Tris buffer containing 150 mM KCl at pH 7.4. The suspension was then transferred under an atmosphere of argon to a screw-cap polycarbonate tube. The lipid suspension was sonicated for 10–15 min in a cup-horn using a Heat Systems-Ultrasonics W-225R sonifier at a power setting of 20. The temperature throughout this period was maintained at 30 C (with the exception of DPPC liposomes, which were maintained at 38 C) using a circulator bath. The sample was then centrifuged for 30 min at 65,000 × g at 4 C. The supernatant was recovered for further use. All procedures were carried out under subdued light. The amount of phospholipid in each preparation was assayed by phosphorus determination (21). Confirmation of the ratio of PC and PE was accomplished by extraction and resolution of the phospholipids via TLC (22), recovery of phospholipids and measurement by phosphorus analysis (21). Cholesterol content was verified by HPLC as detailed.

Peroxidation of liposomes. The three types of liposomal preparations described were incubated in the presence of one of the four oxidizing systems described here. Incubations under these oxidizing conditions were carried out using 2.5 mg of lipid per sample suspended in a total volume of 1.0 ml Tris buffer.

(i) Autoxidation of the liposomes involved dispersing aliquots into aerated Tris buffer followed by incubation in a shaking water bath (80 oscillations/min) for intervals up to 4 hr at 37 C.

(ii) A standard enzymatic O_2^--generating system was used. This involved additions of ADP-Fe^{+3} (prepared by premixing ADP and $FeSO_4$ in water at a ratio of 12:1), where the final concentrations were 120 and 10 μM, respectively. The ADP-Fe^{+2} complex is known to autoxidize rapidly in water yielding ADP-Fe^{+3} (23,24). Incubations were begun by successive additions of 0.50 units XO and 500 μM HX (final concentration). Incubations of these and all other samples were as described for the autoxidation experiments noted above. Under these conditions, O_2^- generation, as measured via cytochrome C reduction (25), remained linear for 15 min at a rate of 1.9 μmol/min and subsided by 20 min. Prolonged incubations (i.e., intervals as long as 3 hr) are presumed to represent oxidations other than those induced by enzymatically generated oxyradicals. The dependence of lipid peroxidation on O_2^- formation was confirmed in preliminary experiments in which >85% of lipid peroxidation was prevented by the addition of 50 μg of SOD. Inhibition of lipid peroxidation by SOD indicates that O_2^- is fulfilling the dual role of maintaining iron in its reduced and catalytically active state and yielding secondary oxidizing species (26).

(iii) Cumene hydroperoxide (200 μM) and 50 μM hematin, prepared as described previously (27), were added to the liposome suspensions. Incubations were initiated by addition of a 100 μl sonicated solution of CuOOH in Tris buffer.

(iv) Irradiation using a ^{60}Co source was carried out by adding 200 μl samples of liposomes to 800 μl of distilled water (pH 7.0) in borosilicate screw-cap test tubes. The tubes were sealed under air or N_2O (20-min purging of samples in a glove bag) prior to subjecting the samples to γ-irradiation. The dose rate of our radiation source was 4.87 Gy/min, and samples were irradiated for set intervals up to 120 min.

Analysis of cholesterol oxidation products. At fixed time intervals, the incubations were terminated by removing 100 μl aliquots for determination of TBAR (28) and immediately thereafter adding 7 vol of chloroform/methanol (2:1, v/v) to the remaining sample. The mixture was briefly centrifuged to separate the organic phase, which was collected and saved. The upper aqueous phase was reextracted as above, and the organic phases were pooled and evaporated under nitrogen. Using this method, >98% of the radioactivity in all samples was extracted.

The dried lipids were suspended in 1.0 ml argon saturated toluene/ethyl acetate (3:2, v/v) and applied to 3-ml solid phase "Diol" extraction columns (Analytichem Corp., Harbor City, CA) conditioned with the same solvent. Eluents were collected under mild vacuum suction,

and the columns were washed with another 2 ml of toluene/ethyl acetate. The combined eluents were immediately evaporated under nitrogen and saved in hexane/benzene (95:5, v/v) under argon at −80 C. Using this technique, >99% of the radioactivity associated with cholesterol or its oxidation products was recovered. The columns were subsequently eluted with methanol to collect the phospholipids. No radioactivity was present in this fraction.

In some experiments, an aliquot of the toluene/ethyl acetate eluent (cholesterol fraction) was removed for TLC as described previously (18). This was done to isolate cholesterol and CT, the latter not being suitably resolved by the HPLC method described below. The identity of CT was confirmed using an authentic nonradiolabeled standard applied along with each sample. The details for recovering and measuring CT by TLC are presented elsewhere (18). The radioactive zones corresponding to cholesterol and CT were scraped from the plates, and the level of radioactivity in each sample was determined by liquid scintillation spectrometry.

The remainder of each cholesterol fraction was reduced to a 100-μl volume, of which 20 μl was subjected to HPLC. HPLC of cholesterol and its oxidation products was performed with a Perkin Elmer Series 4 liquid chromatograph fitted with two 3-μ particle size 100 × 4.6 mm columns (Chromanetics, Inc., Jessup, MD) in series that were eluted isocratically with hexane/isopropanol (95.8:4.2, v/v) at a flow rate of 1.5 ml/min. Detection of peaks was accomplished with an Erma Instruments differential refractometer. The eluent was subsequently delivered to a fraction collector set at 15-sec sampling intervals. The collected samples were then measured for radioactivity by liquid scintillation spectrometry. Using this approach, a radioactivity chromatogram was superimposed on the refractive index chromatogram of authentic cholesterol oxidation standards added to the samples prior to injection. A representative refractive index chromatogram of cholesterol and several oxidation products is shown in Figure 1.

The cholesterol content in each sample was determined following the chromatographic run using an external standard method. Corrections for variations in recovery were based on the radioactive counts in the original sample and the total radioactivity measured after HPLC (i.e., the sum of the radioactivity in all collected fractions). Recoveries ranged from 86 to 96%. The extent of cholesterol oxidation and the amounts of each oxidation product were estimated from the level of radioactivity associated with each peak. Calculations of the amount of each oxidation product were based on the radioactive counts associated with that product expressed as a percentage of the original cholesterol radioactivity. The cholesterol content in each sample was then multipled by the calculated percent for each oxidation product identified yielding a value, in micrograms, for each product formed. This method of calculation assumes that the specific activities of cholesterol and its oxidation products are equal. Verification of each oxidation product identified during the chromatographic run was made by "spiking" radioactive peaks with known amounts of authentic cholesterol oxide standards. The detection limit for most oxidation products by refractometry was 0.5 μg, whereas the limit for peak resolution measured via radioactivity was 3 times background (i.e., 36 dpm).

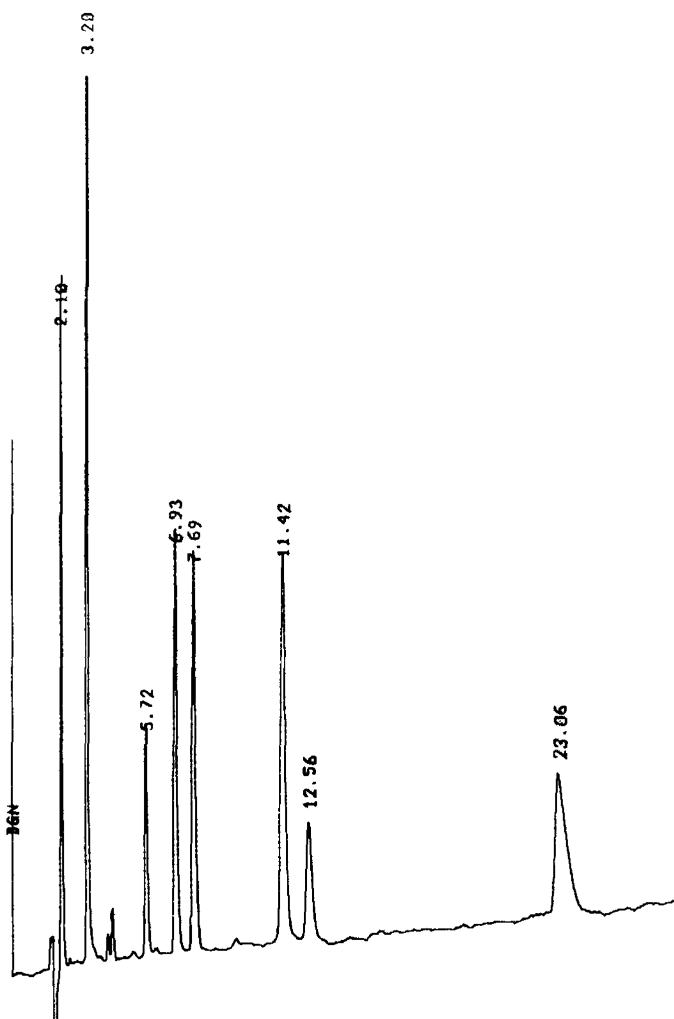

FIG. 1. A high pressure liquid chromatograph of cholesterol oxidation standards. The chromatographic conditions are described in the text. The eluent was monitored by differential refractometry and the integrator response was set at A = 128 for the tracing shown. The oxidation products are indicated by their retention times and are as follows: 2.10, 3,5 diene; 3.20, cholesterol; 5.72, 25-hydroxycholesterol; 6.93, α-CE; 7.69, β-CE; 11.42, 7-keto; 12.56, 7α-OOH; 23.06, 7α-diol.

The extent of cholesterol oxidation was indexed to the level of lipid peroxidation, as measured by TBAR. The level of lipid peroxidation and cholesterol oxidation was determined in all liposome preparations prior to and at various time intervals after the imposed oxidizing conditions already described. All samples were analyzed in duplicate, and values are expressed as the mean and standard deviation calculated from three to four independent experiments for each oxidizing condition described.

RESULTS

The incorporation of cholesterol into liposomes yielded small unilamellar vesicles with characteristics that have been described previously (29,30). The ratio of cholesterol to phospholipid varied no more than 15% from the prescribed ratios at the time of preparation. Furthermore,

TABLE 1

Effect of ^{60}Co Irradiation under N_2O on Cholesterol Oxidation in 1:4 Cholesterol-Phospholipid Liposomes

Duration (min)	Products (in μg)							μmol TBAR[a]
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT	
0	0.072 ±0.009	0.125 ±0.015	0.022 ±0.002	0.147 ±0.025	0.069 ±0.012	0.117 ±0.030	0.102[b]	0.30 ±0.09
30	0.074 ±0.008	0.152 ±0.025	0.029 ±0.007	0.153 ±0.003	0.069 ±0.008	0.081 ±0.014	ND	0.40 ±0.10
60	0.085 ±0.005	0.126 ±0.014	0.024 ±0.007	0.148 ±0.010	0.065 ±0.005	0.084 ±0.015	0.166[b]	0.50 ±0.11
90	0.110 ±0.018	0.160 ±0.025	0.028 ±0.005	0.137 ±0.025	0.064 ±0.010	0.108 ±0.055	0.198[b]	0.49 ±0.15

ND, Not determined. Inadequate chromatography or insufficient numbers of samples prevented accurate determinations of products and quantitation.

[a]The levels of TBAR are expressed as equivalents of malonaldehyde and are calculated from the optical densities measured at 532 nm using a molar extinction coefficient of 1.56×10^4.

[b]Standard deviations not calculated as value represents the average of two measurements.

the type of phospholipid used had no effect on cholesterol assimilation, and the resulting liposomes were stable for the duration of the experiments described. In these experiments, we used either a 1:2 or 1:4 ratio of cholesterol to phospholipid, such that the liposomes differed in terms of the mole percent of cholesterol and degree of unsaturation. The fatty acyl composition of these PC/PE liposomes is described elsewhere (30).

Table 1 shows the major cholesterol oxidation products detected following γ-irradiation of liposomes consisting of a 1:4 molar ratio of cholesterol/phospholipid under an atmosphere of N_2O. In the absence of oxygen, the predominant radical species derived by γ-irradiation of water is ·OH (31). Several oxidation products were measured following irradiation periods as long as 90 min, however, most of these products were present in the original samples (0 min control) and were presumably formed during liposome preparation. This appeared to be the case for all liposome preparations in this report. Cholesterol oxidation was unavoidable during the preparative sonication of liposomes. The levels of oxidation products were low, however, there was some variability in content and proportions of products among different liposome batches. The total amount of cholesterol oxides at zero time were generally less than 0.1% of the total cholesterol content. Accordingly, these products would be undetectable by conventional HPLC methods, and the samples could mistakenly be assumed to be free of oxidized lipids. The only oxidation products to accumulate during γ-irradiation were α-CE and CT. Other cholesterol oxidation products either did not accumulate, or in some cases, decreased below the original levels during the course of irradiation. It should be noted that no TBAR were formed, and levels of 7-OOH remained unchanged over the course of irradiation.

A comparison of lipid peroxidation and cholesterol oxidation in the three liposomal formulations is presented in Table 2 for samples subjected to γ-irradiation under aerobic conditions. Under these conditions, the major oxidizing species formed are O_2^- via the e^-aq, ·OH and H_2O_2 derived from O_2^-. All the cholesterol oxides were found to accumulate in each of the liposomal systems examined. The extent of lipid peroxidation was greatest in the most unsaturated samples (1:4, cholesterol/phospholipid) where the levels of most cholesterol oxidation products and TBAR were as high at 20 min as those formed in 1:2 cholesterol/phospholipid after 60 min of irradiation. Cholesterol oxidation in the cholesterol:DPPC liposomes was least among the three systems studied; however, there was a distinct accumulation of 7-OOH that was not found in the more unsaturated systems. The principal products in all cases were α-CE, β-CE, 7-keto and 3,7-diol. Both α-CE and β-CE were produced in approximately equal proportions during oxidation of cholesterol/DPPC liposomes, whereas the formation of β-CE was favored in liposomes bearing a higher degree of unsaturation. Based on the limited number of lipid peroxidation products analyzed in this study, it appears that product complexity increases during oxidations in the presence of air and, to a lesser extent, as the lipid system becomes more unsaturated.

Subsequent experiments were then conducted using the chemical oxidizing systems. The O_2^- generating system, XO + HX + ADP-Fe^{+3}, is thought to produce a variety of oxidizing species capable of initiating lipid peroxidation (26). The exact species responsible for membrane lipid peroxidation has yet to be determined, although ·OH and perferryl radical are widely viewed as the likely agents. Use of CuOOH plus hematin is representative of lipid peroxide-induced lipid peroxidation. These reactions comprise the propagation stage of lipid peroxidation (14).

Table 3 presents a compilation of data found in Tables 4, 5 and 6, but for the sake of brevity, two oxidizing conditions are selected for comparison. The extent of cholesterol oxidation was found to be influenced by its content in liposomes and by the peroxidizability index (PI) of the component lipids (32). In DPPC/cholesterol liposomes, the only double bonds present are those of

TABLE 2

Effect of Aerobic ^{60}Co Irradiation on Cholesterol Oxidation in Various Cholesterol-Phospholipid Liposomes

Lipid ratio/ duration	Products (in μg)							μmol TBAR[a]
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT	
Chol/PL (1:2)								
0 min	0.10 ±0.04	0.10 ±0.03	0.06 ±0.01	0.05 ±0.01	0.18 ±0.03	0.08 ±0.01	0.04[b]	0.51 ±0.50
2 min	0.26 ±0.08	0.33 ±0.06	0.13 ±0.04	0.10 ±0.03	0.45 ±0.11	0.23 ±0.04	ND	7.96 ±1.29
20 min	0.28 ±0.04	0.38 ±0.07	0.24 ±0.03	0.22 ±0.02	0.50 ±0.12	0.27 ±0.02	ND	10.72 ±2.16
60 min	0.30 ±0.05	0.56 ±0.15	nil	0.37 ±0.02	ND	ND	0.10 ±0.02	32.32 ±2.87
120 min	0.44 ±0.06	0.65 ±0.09	nil	0.55 ±0.10	0.58 ±0.09	0.29 ±0.03	ND	29.11 ±4.97
Chol/PL (1:4)								
0 min	0.22 ±0.02	0.25 ±0.09	nil	0.04 —	0.23 ±0.06	nil	0.06[b]	1.46 ±0.36
2 min	0.32 ±0.02	0.59 ±0.08	ND	0.12 ±0.01	0.50 ±0.10	0.04 ±0.02	ND	22.97 ±4.99
20 min	0.40 ±0.04	0.76 ±0.02	0.08 ±0.02	0.24 0.04	0.51 ±0.08	0.12 ±0.02	0.09[b]	33.22 ±8.05
60 min	0.40 ±0.06	0.79 ±0.04	0.17 ±0.02	0.60 ±0.07	0.88[b]	0.30 ±0.07	ND	46.66 ±2.96
Chol/DPPC (1:4)								
0 min	0.13 ±0.02	0.08 ±0.03	nil	0.04 —	0.21 ±0.05	0.01 —		
60 min	0.36 0.06	0.34 ±0.06	0.20 ±0.05	0.39 ±0.14	0.45 ±0.13	0.12 ±0.01		
120 min	0.50 ±0.11	0.50 ±0.06	0.25 ±0.07	0.54 ±0.10	0.55 ±0.08	0.23 ±0.05		

ND, not determined (see Table 1 for explanation).

[a]The levels of TBAR are expressed as equivalents of malonaldehyde and are calculated from the optical densities measured at 532 nm using a molar extinction coefficient of 1.56×10^4.

[b]Standard deviations not calculated as value represents the average of time measurements.

TABLE 3

Relationship of Cholesterol Oxidation to Lipid Peroxidation in Liposomes of Differing Unsaturated Fatty Acid Content

μmol Conditions	Chol/DPPC (1:4)	Chol/PC:PE (1:2)		Chol/PC:PE (1:4)	
	Total chol. oxidized[b]	Total chol. oxidized[b]	μmol TBAR[a]	Total chol. oxidized[b]	TBAR[a]
Atuox.					
60 min	0.13	0.20	12.82	0.41	15.30
90[c]/120[d] min	0.27	0.24	14.74	0.59	23.00
ADP-Fe^{+3} + XO					
60 min	0.43	ND	ND	0.73	42.94
90[e]/120[f] min	0.62	0.53	46.75	1.02	49.09

ND, not determined. Inadequate chromatography prevented confirmation of species or accurate measurement and quantitation.

[a]The levels of TBAR are expressed as molar equivalents of malonaldehyde and are calculated from the optical densities measured at 532 nm using a molar extinction coefficient of 1.56×10^4. The values shown are the net formation of TBAR obtained by subtracting the levels measured in the starting (unincubated) liposomal preparations from the levels measured at the time intervals indicated.

[b]Values are expressed as percent of cholesterol in the original sample and are the sum of all oxidation products detected.

[c]90-Min incubations were used instead of 120 min for (1:4) Chol/PC:PE liposomes.

[d]120-Min incubations were used for (1:2) Chol/PC:PE and Chol/DPPC liposomes.

[e]90-Min incubations were used for (1:2) and (1:4) Chol/PC:PE liposomes.

[f]120-Min incubations were used for Chol/DPPC liposomes.

TABLE 4

Major Oxidation Products of Cholesterol in Phospholipid Liposomes (1:2 Molar Ratio of Cholesterol to Phospholipid)

Conditions (min)	Products (in μg)						μmol TBAR*
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	
Control							
0	0.08 ±0.03	0.09 ±0.02	nil	0.05 ±0.02	0.04 ±0.01	0.01 —	0.45 ±0.11
Autox.							
10	0.25 ±0.09	0.25 ±0.05	nil	0.14 ±0.03	0.24 ±0.07	nil	1.05 ±0.57
30	0.23 ±0.07	0.31 ±0.10	ND	0.14 ±0.03	0.24 ±0.06	0.02 ±0.006	4.86 ±1.40
60	0.26 ±0.05	0.26 ±0.06	0.01 —	0.23 ±0.04	ND	ND	13.25 ±4.80
90	0.18 ±0.05	0.23 ±0.05	0.05 ±0.01	0.31 ±0.11	0.24 ±0.05	0.06 ±0.02	11.97 ±6.90
120	0.29 ±0.04	0.32 ±0.07	ND	0.35 ±0.05	0.23 ±0.06	ND	15.15 ±5.99
ADP-Fe^{+3} + XO							
10	0.38 ±0.07	0.31 ±0.03	0.26 ±0.07	nil	0.24 ±0.03	nil	15.15 ±0.90
30	0.44 ±0.12	0.49 ±0.12	0.76 ±0.25	0.14 ±0.03	0.21 ±0.06	nil	20.23 ±8.90
90	0.44 ±0.10	0.43 ±0.17	0.35 ±0.20	0.41 ±0.11	0.33 ±0.06	0.03 ±0.009	47.20 ±15.01
CuOOH + hematin							
10	0.20 ±0.02	0.32 ±0.07	0.08 ±0.02	0.13 ±0.04	0.24 ±0.06	nil	6.86 ±0.66
30	0.18 ±0.05	0.36 ±0.09	0.24 ±0.17	0.30 ±0.05	0.33 ±0.06	0.05 ±0.005	22.24 ±4.56
60	0.16 ±0.05	0.29 ±0.07	0.30 ±0.12	0.54 ±0.17	0.36 ±0.13	0.11 ±0.02	28.84 ±3.97
90	0.20 ±0.04	0.37 ±0.09	0.30 ±0.20	0.59 ±0.09	0.37 ±0.11	0.15 ±0.03	33.78 ±12.97
180	0.28 ±0.04	0.44 ±0.11	ND	0.60 ±0.09	ND	ND	35.70 ±14.66

ND, not determined (see Table 1 for explanation).
*, See Table 1 for explanation.

cholesterol, and the PI is accordingly calculated as 0.50. The 1:2 cholesterol/phospholipid liposomes have a PI of 41.1, while the 1:4 cholesterol/phospholipid liposomes have a PI of 65.9. The values shown in Table 3 are expressed as the percentage of total cholesterol oxidized and are estimated from the sum of all cholesterol oxidation products measured. As in Tables 1 and 2, the extent of cholesterol oxidation was least in the DPPC and greatest in the 1:4 cholesterol/phospholipid liposomes. Cholesterol had a suppressive effect on lipid peroxidation, which could be predicted from the slow induction rates for autoxidation (33). Doubling the proportion of cholesterol (1:2 compared to 1:4 liposomes) reduced the level of lipid peroxidation (TBAR) by ca. 20%. Although a lower percentage of cholesterol was oxidized in the 1:2 liposomes, the actual amounts of cholesterol oxidation products were similar to that produced following oxidation of 1:4 liposomes. Thus, the dcrease in TBAR formation in the 1:2 cholesterol/phospholipid liposomes was accompanied by an increase in the amounts of cholesterol oxidized. It should be noted that a similar extent of cholesterol oxidation was achieved following 60-min treatments with ADP-Fe^{+3} + XO and 60 min of γ-irradiation in air.

The product profiles for the $O_2^{\overline{\cdot}}$-dependent and CuOOH-induced peroxidations are shown in Tables 4 through 6. During the initial periods of oxidation (<30 min), these oxidizing systems yielded remarkably different proportions of cholesterol oxidation products, however, with prolonged incubations the profile of oxidation products began to resemble each other. α-CE and β-CE were formed in approximately equal amounts during the early intervals of oxidation. As the incubation period progressed, the relative amounts of β-CE increased, and it became a major product, particularly in unsaturated lipid systems. In liposomes bearing unsaturated fatty acids, 7-OOH was an early oxidation product that gradually accumulated.

TABLE 5

Major Oxidation Products of Cholesterol in Phospholipid Liposomes (1:4 Molar Ratio of Cholesterol to Phospholipid)

Conditions (min)	Products (in μg)							μmol TBAR*
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT	
Control								
0	0.08 ±0.01	0.10 ±0.03	0.03 —	0.04 ±0.01	0.08 ±0.02	0.02 —	0.06 ±0.01	1.09 ±0.47
Autox.								
10	0.25 ±0.05	0.25 ±0.03	0.05 ±0.01	0.05 ±0.01	0.28 ±0.09	ND	ND	8.50 ±1.38
30	0.26 ±0.07	0.27 ±0.06	0.05 ±0.01	0.15 ±0.05	0.33 ±0.05	0.04 ±0.01	ND	11.70 ±2.12
60	0.28 ±0.04	0.28 ±0.06	0.06 ±0.02	0.18 ±0.04	0.29 ±0.07	0.06 ±0.02	0.10 ±0.07	17.47 ±5.79
90	0.36 ±0.06	0.49 ±0.06	ND	0.18 ±0.02	0.35 ±0.06	0.09 ±0.03	0.15 ±0.05	25.16 ±11.00
ADP-Fe^{+3} + XO								
10	0.26 ±0.04	0.26 ±0.03	0.20 ±0.08	0.19 ±0.02	0.25 ±0.06	ND	ND	18.17 ±1.03
30	0.27 ±0.09	0.36 ±0.09	0.19 ±0.06	0.19 ±0.05	0.34 ±0.03	0.13 ±0.04	0.09 ±0.02	39.62 ±9.42
60	0.31 ±0.09	0.57 ±0.13	0.23 ±0.02	0.34 ±0.07	0.35 ±0.12	0.10 ±0.04	0.09 ±0.01	45.03 ±12.96
90	0.33 ±0.06	0.62 ±0.08	0.19 ±0.08	0.43 ±0.08	0.34 ±0.05	0.14 ±0.04	ND	51.18 ±14.27
CuOOH + hematin								
10	0.16 ±0.02	0.26 ±0.03	0.18 ±0.07	0.25 ±0.03	0.40 ±0.06	0.04 0.01	ND	25.16 ±6.01
30	0.27 ±0.08	0.59 ±0.14	0.23 ±0.05	0.72 ±0.16	0.73 ±0.15	0.08 ±0.02	0.24 ±0.06	59.69 ±8.02
60	0.44 ±0.11	0.79 ±0.19	0.22 ±0.05	0.94 ±0.26	0.88 ±0.16	0.16 ±0.02	0.46 ±0.04	76.96 ±16.97

ND, not determined (see Table 1 for explanation).
*, See Table 1 for explanation.

TABLE 6

Major Oxidation Products of Cholesterol in Phospholipid Liposomes (1:4 Molar Ratio of Cholesterol to Dipalmitoylphosphatidylcholine)

Conditions	Products (in μg)						
	α-CE	β-CE	7-OOH	7-keto	3,7-diol	3,5-diene	CT
Control							
0 min	0.03 —	nil	0.05 ±0.01	0.03 ±0.005	0.10 ±0.04	nil	nil
Autox.							
60 min	nil	nil	0.09 ±0.02	ND	0.31 ±0.13	nil	nil
120 min	0.08 ±0.04	0.10 ±0.04	0.09 ±0.02	0.12 ±0.03	0.32 ±0.07	ND	ND
ADP-Fe^{+3} + XO							
60 min	0.11 ±0.04	0.14 ±0.05	0.15 ±0.05	0.20 ±0.03	0.26 ±0.12	0.11 ±0.02	0.09 ±0.03
120 min	0.13 ±0.02	0.16 ±0.05	0.25 ±0.02	0.28 ±0.07	0.35 ±0.07	0.18 ±0.04	0.09 ±0.04

In saturated liposomes, the extent of 7-OOH accumulation was as great as any of the other products measured. Under virtually all conditions, 7-keto and, to a lesser extent, 3,7-diol were major products that formed with prolonged incubations. Small but consistently detectable amounts of 3,5-diene were found under these conditions, however, its content relative to other products was greatest in the DPPC/cholesterol liposomes.

The relative proportions of cholest-5-ene-3β,7β-diol and cholest-5-ene-3β,7α-diol (the 3,7-diols) and the corresponding 7α- and 7β-hydroperoxides (7-OOH) could not be established with certainty due to the limited resolution of these isomers by HPLC. Approximation of the levels of radioactivity associated with each peak indicated that equal amounts of each isomer were formed under all circumstances. These approximations are subject to error of 25 to 35% due to peak overlap. We also note the absence of 25-hydroxycholesterol formation under all the oxidizing conditions studied.

DISCUSSION

The characterization of cholesterol oxidation products following free radical-induced membrane lipid peroxidation was studied using unilamellar liposomes. These artificial membranes permitted the manipulation of lipid composition which in this study involved the formulation of discrete proportions of unsaturated phospholipids and cholesterol. Based upon total lipid content the amount of cholesterol added to the liposomes was similar to the proportions found in cell membranes. For example, the plasma membrane of hepatocytes contains ca. 12.9% cholesterol by weight (34,35), while this percentage is lower in endoplasmic reticulum (3.3%) (35) and mitochondria (1.5%) (36). In some cases, the proportions of cholesterol may be even higher than those cited above (37).

It is possible to subdivide the cholesterol oxidation products identified in this study into two categories based on whether lipid peroxidation was mediated by oxyradical-dependent initiation reactions or by lipid peroxide-dependent propagation reactions. Liposomes subjected to anaerobic γ-irradiation represented one extreme, where cholesterol is probably oxidized by ·OH attack and the likelihood for lipid peroxidation chain reactions is low. Under these conditions the only products to accumulate were α-CE and CT (Table 1). γ-Irradiation in the presence of air (or O_2^-· generation via xanthine oxidase) yields a more complex array of oxyradicals, however, the principal oxidants are likely to be ·OH (or a perferyl radical) and H_2O_2, since O_2^-· has been shown to be incapable of directly oxidizing cholesterol (38). The presence of oxygen also permits oxidations to proceed via propagation reactions. The degree of propagation reactions may be limited either by short incubation intervals or more effectively by minimizing the degree of lipid unsaturation. The variety and amounts of cholesterol oxides formed are also increased with either prolonged irradiation or increased lipid unsaturation. Under these conditions, propagation reactions largely contribute to the accumulation of lipid peroxidation products, measured as TBAR and a host of cholesterol oxides. A comparison of data from Tables 1 and 2 indicates that in the absence of lipid peroxidation the yields of cholesterol oxidation products are low, where net formation of only two products is seen. By contrast, provision of oxygen permits considerable lipid peroxidation and an increased number of cholesterol oxidation products.

The free radical reactions involved in lipid peroxidation appear to be reflected by the types of cholesterol oxidation products. For example, in the absence of 7-OOH most of the other oxidation products are not encountered. There is, however, one important exception to this, i.e., α-CE formation after anaerobic γ-irradiation occurs in the absence of other products (Table 1). This indicates that 7-OOH are not produced under anaerobic conditions via ·OH, and, accordingly, no derived products are encountered. The absence of peroxidative chain reactions is expected during anaerobic irradiation and should be suppressed in a largely saturated lipid matrix such as the DPPC/cholesterol liposomes. However, a variety of cholesterol oxidation products are found after aerobic oxidation of DPPC/cholesterol liposomes, indicating the involvement of oxyradical initiation reactions. We note that in saturated liposomes subjected to aerobic oxidations, 7-OOH accumulates as a significant product (Tables 2 and 6), in agreement with the findings of Muto et al. (11). It is plausible that 7-OOH is more stable in saturated lipids where its decomposition is determined largely by interactions with other cholesterol molecules. Products such as 7-keto, 3,7-diol and the isomeric cholesterol epoxides may arise by reactions of 7-OOH with itself (39) or with O_2^- where both 7-OOH and O_2^- are generated by the aerobic oxidations described.

Lipid peroxidation via CuOOH plus hematin can be contrasted from the initiation-type reactions discussed above. Peroxidation was rapidly induced following addition of CuOOH, and the profile of cholesterol oxidation products after short incubation intervals (≤30 min) resembled those obtained after longer incubations (≥60 min) with the O_2^-·-generating system. Under otherwise similar conditions, peroxide-dependent (CuOOH) oxidations produce a more rapid accumulation of TBAR, 7-OOH and derived products 7-keto and 3,7-diol, than O_2^--dependent oxidations. During the first 10 min of O_2^--dependent oxidation (Table 4 and 5), less oxidation products form, and with the exception of the 3,5-diene, approximately equal amounts of each product are detected. Beyond 30 min (when O_2^- is no longer being generated), the pattern of oxidation changes considerably and the cholesterol epoxides, 7-keto and 3,7-diols predominate. The oxidation profile resembles that obtained by autoxidation (although the amounts of each product are greater under these conditions of imposed oxidation), suggesting that lipid peroxidation is proceeding via autoxidation at these later incubation periods.

The yields of the α- vs β-CE are indicative of the prevailing process of cholesterol oxidation. Both isomers form under limited oxyradical-induced peroxidation. It appears that γ-irradiation under aerobic conditions or peroxidation via O_2^-· give rise to similar levels of epoxide isomers. Extensive peroxidation, assessed by high TBAR levels, clearly favors β-CE formation. In this respect, peroxyl species have been shown to mediate cholesterol oxidation by lipoxygenase (40) with marked β-stereoselectivity toward the cholesterol hydroperoxide and epoxide products. Gumulka et al. (41) proposed that the proportions of α- and β-CE provide a clue to the processes involved in cholesterol oxidation. They observed a predominance of β-CE following autoxidation, lipid peroxidation and

ozonization, whereas α-CE was preferentially formed by one-atom oxidants such as epoxidases and peracids. This contention is verified by our observations, and although the ratios of α- to β-CE in this study are lower than those reported previously (8,39,41,42), the directions of change are similar.

The formation of the isomeric epoxides by oxidation of cholesterol with H_2O_2 and other organic peroxo-compounds has been reported (8). Cholesterol epoxidation by reaction with hydroperoxides has been documented in organic solvents, aqueous dispersions (11,39,43), and considerable epoxidation also takes place when cholesterol is added to dispersions of lipid hydroperoxides isolated from hepatic microsomes (44). In each of these cases β-CE formation was predominant. Membranes consisting of only saturated fatty acids (Tables 2 and 6) may support cholesterol oxidation via oxyradicals, but cannot effectively produce lipid peroxides and sustain propagation reactions. The oxidation reactions are, therefore, limited to direct attack by oxyradicals (as in O_2^--dependent initiation of lipid peroxidation) or to interactions between oxidized cholesterol radicals. This results in a much reduced level of cholesterol oxidation and is accompanied by the formation of equal amounts of α- and β-CE. γ-Irradiation under anaerobic conditions (Table 1), or irradiation of DPPC/cholesterol liposomes in the presence of oxygen (Table 2), also yield equal amounts of α- and β-CE.

Based on these findings, we submit that oxyradical-induced cholesterol oxidation resembles the one-atom oxidants previously noted. These "initiation" reactions likely proceed by radical attack of the α-surface of cholesterol, while attack by peroxo-compounds (e.g., fatty acid hydroperoxides of phospholipids) preferentially involves the β surface, as originally proposed by Teng and Smith (2). The isomeric selectivity of oxyradicals vs peroxyl-mediated epoxidations may be explained on the basis of cholesterol orientation in membranes. According to Vandenheuval (45), cholesterol is embedded into the membrane bilayer with its α surface facing and at a 5–6 Å distance from the glycerol-phosphate backbone of an adjacent phospholipid. This places the α surface of the 5,6 double bond in relative proximity to oxyradicals generated in the aqueous phase, however, the radical species responsible for epoxidation remains to be identified. The β surface, on the other hand, is oriented toward adjacent fatty acyl groups facing the membrane interior. Attack by oxyradicals would be hindered by the proximate C-19 methyl group of cholesterol and the hydrophobic environment surrounding the β surface of the 5,6 double bond. In this arrangement the double bond is estimated to be 4–5 Å from an ω-3 double bond of a neighboring polyunsaturated fatty acid. Lipid peroxidation involving these polyunsaturated fatty acids would thus favor reaction of the peroxides with the β surface of cholesterol.

In addition to cholesterol epoxide formation, considerable amounts of CT and 3,5-diene were detected. The only known source for CT is the isomeric cholesterol epoxides, and under the conditions used in this study, CT could only be formed by spontaneous hydrolysis of the epoxides. However, the previously reported rates for spontaneous hydrolysis of either epoxide isomer (18,46) appear to be too slow to produce the amounts of CT detected. Nevertheless, an accumulation of CT would be consistent with cholesterol oxidation in the wake of lipid peroxidation and cholesterol epoxide formation. Formation of 3,5-diene has been proposed to derive from 7-OOH via dehydration of 7-keto (47). It appears that accumulation of 3,5-diene, relative to other products, is favored under conditions where 7-OOH can undergo decomposition without participating in further chain reactions. These conditions prevail in a saturated lipid matrix (Table 6) or under anaerobic conditions (Table 1).

Our findings are in general accordance with those of Terao et al. (10) and Muto et al. (11). They concluded that cholesterol was cooxidized with unsaturated fatty acids and that peroxyl and alkoxyl radicals derived from unsaturated fatty acids were largely responsible for the generation of the cholesterol oxides. The 7-keto and 3,7-diol products, also found as major products in these earlier studies, were proposed to derive from thermolytic decomposition of 7-OOH. The other major products were the isomeric cholesterol epoxides, which were proposed to arise from alkoxyl and peroxyl-radical attack of cholesterol in a process showing marked β-stereoselectivity (11).

Lipid peroxidation in tissues is expected to be minimized by a plethora of antioxidants and scavengers that prevent both radical initiation and chain reactions. The detection of low levels of lipid peroxidation products in living systems may be considered proof for low-level free radical reactions. If this is indeed the case, then the cholesterol oxidation profile from biological systems should resemble the oxyradical profiles described in this report. Based on cholesterol epoxide content, in vivo lipid peroxidation should be accompanied by approximately equal β- vs α-CE ratios. Available evidence, though sparse (19,48,49), supports this hypothesis. It may be possible to examine the course of lipid peroxidation in more complex systems containing cholesterol by "finger-printing" the oxidation products in a manner similar to that described in this report.

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Liver Subcellular Fatty Acid Profiles of Chicks Fed Diets Containing Hydrogenated Fats and Varying Linoleate Levels[1]

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Day-old male broiler chickens were fed semipurified diets containing 5% lipid from one of four different lipid sources: corn oil (CO), partially hydrogenated soybean oil (HSBO), a spent restaurant grease (SRG) and a purified mixture of triolein, tripalmitin and tristearin (OPS). Diets CO and HSBO contained adequate amounts of linoleic acid, but diets SRG and OPS were deficient in linoleate. In addition, SRG and HSBO contained *trans* isomers of 16:1 and 18:1. The diets were fed for 3 wk to determine the effects of low linoleate levels and *trans* isomers on fatty acid profiles in liver microsomes, mitochondria and cytosol. Chicks fed HSBO had the highest body weights, while those fed SRG and OPS had the lowest. The incidence and severity of dermatitis were similar for all treatments. The proportions of linoleate and arachidonate in lipids from liver subcellular fractions were reduced significantly in chicks fed OPS and SRG; however, levels of 20:3ω9 were not increased. Feeding HSBO, which is high in both linoleate and linolenate, resulted in higher levels of 18:3ω3 and 20:5ω3 in liver subcellular fractions and lower levels of 20:4ω6 than those seen in chicks fed CO. The isomeric forms of 18:1 present in the partially hydrogenated fats (HSBO and SRG) appeared to be incorporated into the lipids of liver fractions. The results of this study show that dietary lipids influence fatty acid profiles of chick liver microsomes, mitochondria and cytosol. Decreases in linoleate and arachidonate in these organelles occur before overt essential fatty acid (EFA) deficiency signs in chicks fed EFA-deficient diets.
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Hydrogenation of vegetable and marine oils to increase plasticity and improve shelf life results in a decrease in polyunsaturated fatty acids (PUFA) and the formation of positional and geometric isomers, particularly of octadecenoic acid (1). As much as 20% of the fatty acids in hydrogenated vegetable oils are *trans* isomers of octadecenoic acid, while approximately 25% are *cis* isomers with the double bond located at the C4 to C16 positions (2,3).

Feed-grade blended fats make up nearly half of the 8 $\times 10^8$ kg of fats fed to food animals. In 1985, nearly 40% of the blended fats were restaurant greases (Rouse Marketing, personal communication). These fat sources contain large amounts of *cis/trans* positional isomers of 18:1 and lesser amounts of 18:2 isomers, which may be incorporated into animal products.

The composition of dietary lipids can influence not only the depot fat in adipose tissue (4), but also the phospholipids of intracellular organelles of various tissues (5). Many studies have shown that positional and geometric isomers of octadecenoic acid are readily absorbed and incorporated into tissue lipids of rats and humans (1,2,6,7). *Trans* isomers are incorporated into phospholipids (3,8) and may interfere with the biosynthesis of PUFA in rat liver microsomes by inhibiting Δ9, Δ6 and Δ5 desaturase enzymes (9,10). Certain *cis* isomers of octadecenoic acid are preferentially incorporated into phospholipids and may interfere with desaturation and elongation of fatty acids (2,3).

When fed in conjunction with essential fatty acid (EFA)-deficient diets, partially hydrogenated vegetable oils intensify the deficiency symptoms of poor growth and dermatitis in rats (10). In vitro studies on the inhibition of liver microsomal desaturases from EFA-deficient rats by positional and geometric isomers of octadecenoic acid show that the degree of inhibition is influenced by the position of the double bond (9,11).

Though many studies on the effects of hydrogenated oils on lipid metabolism in the presence and/or absence of EFA deficiency have been done in the rat, none have been found using the chicken. The present study was undertaken to determine the effects of feeding partially hydrogenated fats and/or diets deficient in EFA on the composition of lipids in liver subcellular organelles of young growing chicks.

MATERIALS AND METHODS

Four experimental diets were prepared from the basal diet described in Table 1. Each diet contained 50 g/kg of one of the following lipid sources: corn oil (CO), spent restaurant grease (SRG), partially hydrogenated soybean oil (HSBO) and a triglyceride mixture containing 30% triolein, 30% tripalmitin and 40% tristearin (OPS). SRG, obtained from a local restaurant, was composed of 80% prime tallow and 20% hydrogenated cottonseed oil. Further hydrogenation and isomerization was expected as a result of heating. HSBO (a margarine) contained a mixture of liquid soybean oil and partially hydrogenated soybean oil with added lecithin and mono- and diglycerides. Purified triglycerides were purchased from United States Biochemical Corp. (Cleveland, OH).

Day-old male broiler chicks (Hubbard strain) were randomly assigned to the four experimental diets. Each dietary treatment contained two replicate pens of eight chicks. The chicks were maintained in Petersime batteries and exposed to a cycle of 18 hr light and 6 hr dark for the duration of the 21-day experiment. Feed and water were provided ad libitum throughout the study. Total feed consumption and body weight gains were determined at the completion of the experiment for calculation of feed conversion (total feed/total gain).

On day 21, chicks were weighed and scored for perosis and dermatitis on a scale of 0 to 4 (12). Four chicks from

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Abbreviations: EFA, essential fatty acid; OPS, mixture of triolein, tripalmitin and tristearin used as dietary fat source; CO, corn oil; SRG, spent restaurant grease; HSBO, partially hydrogenated soybean oil; PUFA, polyunsaturated fatty acids.

TABLE 1

Composition of Basal Diet

Ingredient	Amount (g/kg diet)
Isolated soy protein[a]	250.0
Corn starch	561.0
Fat[b]	50.0
Dried egg albumen[c]	30.0
Mineral mix[d]	30.0
Cellulose	30.0
$CaHPO_4.H_2O$	25.0
$CaCO_3$	15.0
Vitamin premix[e]	5.0
DL-Methionine	4.0
Calculated nutrient analysis	
Metabolizable energy	3400 kcal/kg
Crude protein	246.5 g/kg

[a]RP 101, crude protein, 90%, Ralston Purina Co., Richmond, IN.

[b]One of the following fat sources: corn oil (CO), spent restaurant grease (SRG), hydrogenated soybean oil (HSBO) or triolein, tripalmitin, tristearin mix (OPS).

[c]Dried egg albumen, Henningsen Foods, 14334 Industrial Rd., Omaha, NE.

[d]Contains (in mg/kg of diet) $CoCl_2.6H_2O$, 5; $CuSO_4.5H_2O$, 60; $FeSO_4.7H_2O$, 500; KCl, 1500; K_2HPO_4, 6000; KIO_3, 6; $MgSO_4.7H_2O$, 6000; Na_2SeO_3, 0.43; ZnO, 90.

[e]Contains (per kg of diet) vitamin A, 4500 IU; vitamin D_3, 4500 ICU; vitamin E, 50 IU; vitamin K, 1.5 mg; thiamin, 15 mg; riboflavin, 15 mg; calcium pantothenate, 15 mg; niacin, 50 mg; pyridoxine, 6 mg; folic acid, 6 mg; vitamin B_{12}, 0.02 mg; biotin, 200 μg; choline, 1 g.

each experimental treatment (two per pen) were killed by cervical dislocation, and the livers were quickly removed. A 3-g sample of each liver was taken for subcellular fractionation and placed in a beaker containing ca. 10 ml chilled 0.25 M sucrose.

Livers were homogenized in the 0.25 M sucrose solution with a Kinematica Polytron (Luzern, Switzerland), and subcellular fractions were isolated by modifying the method of Mayne and Parker (13). The homogenate was centrifuged at 750 × g for 10 min to remove cellular debris and nuclei. The supernatant was centrifuged at 10,000 × g for 10 min to obtain a mitochondrial pellet, which was washed with 0.25 M sucrose, resuspended by homogenation and recovered after a second centrifugation at 10,000 × g. The supernatant containing cytosol and microsomes was centrifuged at 100,000 × g for 60 min to separate these components. All centrifugations were performed at 0 C, and samples were kept on ice between centrifugations. Mitochondrial and microsomal pellets, as well as samples of cytosol, were frozen (−20 C) until needed for fatty acid analysis.

Total lipids from subcellular fractions were extracted in chloroform/methanol (2:1, v/v) by the method of Bligh and Dyer (14). Methyl esters were prepared after saponification using boron trifluoride (15). The methyl esters were extracted in isooctane for chromatographic analysis using a HP 5890 gas chromatograph equipped with a flame ionization detector and a Nelson Analytical Data System (Hewlett Packard Co., Sunnyvale, CA). A DB225 (25% cyanopropylphenyl) fused silica megabore column (30 m × 0.53 mm ID) purchased from J & W Scientific Co. (Rancho Cordova, CA) was used with helium as a carrier gas at a flow rate of 3.4 ml/min. The oven temperature was maintained at 200 C for the first 15 min of the run and increased at a rate of 0.8 C per min to a final temperature of 218 C. Total analysis time per sample was 55 min. Both injector port and detector were set at 250 C.

Fatty acid standards for gas chromatography were purchased from Nu-Chek-Prep, (Elysian, MN). Proportions of fatty acids present in each sample were determined as area percent.

Fatty acid analysis was also done for each dietary lipid source, and the ratios of *cis* to *trans* isomers of 16:1 and 18:1 in SRG and HSBO were determined in triplicate by combined argentation (16) and gas chromatography. Silver nitrate-impregnated thin layer chromatography (TLC) plates were prepared by immersing precoated TLC plates (Silica Gel G) into a saturated methanolic solution of $AgNO_3$. Methylated samples of each lipid were applied, and the plates were developed twice in hexane/diethyl ether/glacial acetic acid (94:4:2, v/v/v). Methyl nonadecanoate was added as an internal standard to the recovered fractions, which were then analyzed by gas chromatography as described above.

Data were analyzed statistically by analysis of variance. When sample sizes were equal Tukey's post-priori test was used, and when sample sizes were unequal Bonferroni's multiple comparison post-priori test was employed (17). Variation between means was expressed as a pooled SEM for fatty acid data when group sample sizes were equal. A pooled SD was used when sample sizes were unequal.

RESULTS

The major fatty acids found in the four dietary lipids are given in Table 2. The triglycerides used in the OPS mixture were not pure; the triolein sample contained 69.0%

TABLE 2

Fatty Acid Composition (Area %) of Dietary Lipids

Fatty acid	Dietary lipid source[a]			
	CO	SRG	OPS	HSBO
14:0	3.32	4.13	5.47	0.00
14:1	0.00	0.72	0.27	0.00
15:0	0.00	0.69	0.10	0.00
16:0	12.53	28.15	31.55	11.42
16:1[t]	0.00	0.18*	0.00	0.00
16:1[c]	0.11	2.76*	1.60	0.00
17:0	0.08	1.60	0.05	0.12
18:0	2.34	16.77	35.10	8.04
18:1[t]	0.00	6.37*	0.00	17.16*
18:1[c]	26.37	30.05*	20.68	23.53*
18:2ω6[tt]	0.00	0.36	0.10	1.01
18:2ω6[cc]	53.29	3.64	2.18	31.67
18:3ω6	0.05	0.03	0.03	0.13
18:3ω3	0.84	0.13	0.02	4.19
20:0	0.43	0.14	0.25	0.31
% kcal of ME as linoleate in diets	7.04	0.48	0.28	4.18

[a]CO, corn oil; SRG, spent restaurant grease; OPS, triolein, tripalmitin and tristearin; HSBO, partially hydrogenated soybean oil.
*Contains positional isomers.

TABLE 3

Body Weights (at 21 days), Perosis and Dermatitis Scores and Feed Conversions of Chicks Fed the Experimental Diets

Fat source	n	Average body weight* (g)	Perosis	Dermatitis	F/G†
CO	13	469[a,b]	0.62	0.27	1.26[c]
SRG	15	400[b,c]	0.00	0.37	1.33[b]
OPS	15	385[c]	0.13	0.40	1.41[a]
HSBO	16	510[a]	0.06	0.38	1.31[b]
Pooled SD		72.6	0.68	0.57	0.01

*CO, Corn oil; SRG, spent restaurant grease; OPS, triolein, tripalmitin and tristearin; HSBO, partially hydrogenated soybean oil. Different superscripts denote significant differences between group means as determined by Bonferroni's multiple comparison post-priori test, $p < 0.05$.

†Total feed/total gain. Different superscripts denote significant differences between group means (two pens/treatment) as determined by Tukey's post-priori test, $p < 0.05$.

TABLE 4

Liver Microsomal Fatty Acids (Area %) of Chicks Fed the Experimental Diets

	Dietary lipid				
Fatty acid	CO	SRG	OPS	HSBO	Pooled SEM
16:0	24.54	24.61	26.37	24.37	1.05
16:1	3.13[b]	7.84[a]	7.10[a,b]	5.40[a,b]	1.07
18:0	20.06	16.17	17.28	18.51	1.06
18:1	21.63[b]	30.77[a]	29.17[a]	27.01[a,b]	1.64
18:2ω6	13.77[a]	7.83[b]	7.95[b]	12.39[a]	0.66
18:3ω6	0.25[a]	0.25[a]	0.13[b]	0.20[a,b]	0.03
18:3ω3	0.09[b]	0.15[b]	0.13[b]	0.26[a]	0.02
20:2ω6	0.97[b]	1.67[a,b]	2.12[a]	1.03[b]	0.24
20:3ω9	0.25[a]	0.14[b]	0.19[a,b]	0.18[a,b]	0.02
20:3ω6	2.18	1.61	1.62	1.99	0.18
20:4ω6	7.61[a]	3.40[b]	4.07[b]	4.42[b]	0.54
20:5ω3	0.09[c]	0.39[b]	0.29[b]	0.52[a]	0.03
22:4ω6	1.06	0.24	0.24	0.19	0.22
22:5ω6	1.18[a]	0.20[b]	0.21[b]	0.11[b]	0.16
22:5ω3	0.00	0.00	0.04	0.15	0.05
22:6ω3	0.52	0.66	0.38	0.97	0.21
Total ω3	0.71[b]	1.20[a,b]	0.84[b]	1.90[a]	0.22
Total ω6	27.03[a]	15.19[b]	16.34[b]	20.33[b]	1.55
ω6/ω3	38.35	13.11	25.27	11.01	
18:2/20:4	1.86	2.33	1.98	2.81	

CO, corn oil; SRG, spent restaurant grease; OPS, triolein, tripalmitin, tristearin; HSBO, partially hydrogenated soybean oil. Different superscripts within a row indicate significant differences between group means ($p < 0.05$).

oleate (smaller amounts of 14:0, 16:0, 16:1 and 18:2) while the tripalmitin and tristearin samples were 99.7% 16:0 and 86.7% 18:0, respectively. Both the OPS and SRG diets were deficient in linoleic acid. The OPS diet provided 1.09 g linoleate/kg diet, and the SRG diet provided 1.82 g linoleate/kg diet. This is far below the 10 g/kg recommended by the National Academy of Science (18). In contrast, the HSBO and CO diets provided more than 15 g linoleic acid/kg diet.

Body weights at 21 days were significantly influenced by dietary fat source (Table 3). Chicks fed CO and HSBO were heavier ($p < 0.05$) than those fed OPS, while weights of chicks fed SRG were not different from those of chicks fed CO or OPS. The poorest feed conversion was observed in chicks fed OPS, and the best was seen in those fed CO. Perosis and dermatitis were observed in chicks from each treatment, but no significant effects due to EFA deficiency were noted.

The fatty acid profiles for microsomes, mitochondria and cytosol are presented in Tables 4–6. The DB225 megabore column used for chromatographic analysis of fatty acids in this experiment permitted baseline separation

TABLE 5

Liver Mitochondrial Fatty Acids (Area %) of Chicks Fed the Experimental Diets

	Dietary lipid				
Fatty acid	CO	SRG	OPS	HSBO	Pooled SEM
16:0	27.43	26.12	29.70	27.62	1.35
16:1	3.44[b]	9.16[a]	7.84[a,b]	6.23[a,b]	1.12
18:0	18.44[a]	14.88[a,b]	14.06[b]	16.09[a,b]	0.97
18:1	20.54[b]	29.23[a]	30.91[a]	26.82[a]	1.12
18:2ω6	14.97[a]	8.32[b]	7.38[b]	12.24[a]	0.91
18:3ω6	0.34[a]	0.26[a,b]	0.12[b]	0.20[a,b]	0.04
18:3ω3	0.15	0.18	0.20	0.20	0.07
20:2ω6	0.79	0.97	1.26	0.75	0.15
20:3ω9	0.32[a]	0.05[b]	0.09[b]	0.08[b]	0.04
20:3ω6	1.58[a]	0.86[b]	0.98[a,b]	1.27[a,b]	0.16
20:4ω6	6.79[a]	3.12[b]	2.98[b]	4.10[b]	0.63
20:5ω3	0.07[c]	0.38[a,b]	0.20[b,c]	0.44[a]	0.05
22:4ω6	0.38	0.07	0.13	0.10	0.08
22:5ω6	0.67[a]	0.15[b]	0.17[b]	0.08[b]	0.06
22:5ω3	0.02	0.07	0.02	0.08	0.04
22:6ω3	0.27	0.51	0.40	0.60	0.12
Total ω3	0.51[b]	1.14[a,b]	0.82[a,b]	1.32[a]	0.18
Total ω6	25.53[a]	13.75[b]	13.03[b]	18.73[b]	1.55
ω6/ω3	72.74	13.03	15.84	15.51	
18:2/20:4	2.28	3.01	2.51	3.02	

CO, corn oil; SRG, spent restaurant grease; OPS, triolein, tripalmitin, tristearin; HSBO, partially hydrogenated soybean oil. Different superscripts within a row indicate significant differences between group means ($p < 0.05$).

TABLE 6

Liver Cytosol Fatty Acids (Area %) of Chicks Fed the Experimental Diets

	Dietary lipid				
Fatty acid	CO	SRG	OPS	HSBO	Pooled SEM
16:0	34.64	28.31	33.02	30.46	1.88
16:1	5.66	9.60	8.74	7.81	1.44
18:0	14.14	11.56	10.75	11.43	1.04
18:1	29.52[b]	38.72[a]	36.17[a,b]	35.50[b]	1.51
18:2ω6	9.55[a]	4.97[b,c]	4.41[c]	7.31[a,b]	0.62
18:3ω6	0.16	0.10	0.12	0.13	0.04
18:3ω3	0.06[b]	0.10[a,b]	0.06[b]	0.28[a]	0.04
20:2ω6	0.30[b]	0.94[a]	0.83[a,b]	0.47[a,b]	0.15
20:3ω9	0.02	0.00	0.04	0.04	0.02
20:3ω6	0.86	0.81	0.57	0.81	0.12
20:4ω6	2.98[a]	1.74[a,b]	1.39[b]	1.79[a,b]	0.36
20:5ω3	0.00[b]	0.16[a]	0.11[a,b]	0.24[a]	0.03
22:4ω6	0.07	0.02	0.02	0.11	0.04
22:5ω6	0.08	0.05	0.04	0.04	0.05
22:5ω3	0.13	0.08	0.02	0.10	0.08
22:6ω3	0.21	0.31	0.20	0.40	0.10
Total ω3	0.40	0.64	0.39	1.01	0.20
Total ω6	14.00[a]	8.64[b]	7.37[b]	10.67[a,b]	1.10
ω6/ω3	41.56	13.07	19.50	10.66	
18:2/20:4	3.38	3.02	3.23	4.12	

CO, corn oil; SRG, spent restaurant grease; OPS, triolein, tripalmitin, tristearin; HSBO, partially hydrogenated soybean oil. Different superscripts within a row indicate significant differences between group means ($p < 0.05$).

of 18:2ω6 *cis* and *trans* isomers and near-baseline resolution of 18:1ω9 *cis* and *trans* isomers. However, the many positional isomers of 18:1 in SRG and HSBO could not be separated, and only one large nonsymmetrical peak and a small incompletely resolved peak after the major 18:1 peak were eluted. The areas of these two peaks were combined for statistical analysis. Although isomeric separation was incomplete, the shape of the 18:1 peaks found in liver subcellular components mimicked the shape of the 18:1 peaks found in the dietary fat source, suggesting that dietary 18:1 isomers were incorporated into liver cells. Unresolved positional isomers were present in both the *cis* and *trans* 18:1 fractions of SRG and HSBO separated by argentation chromatography.

Feeding EFA-deficient diets (SRG and OPS) led to a significant increase in the proportions of 18:1 and 20:2ω6 in microsomes (Table 4) and a decrease in the proportion of 18:2ω6, 18:3ω6 and 22:5ω6 when compared to the CO control. Chicks fed HSBO had increased proportions of 18:3ω3 and 20:5ω3 and decreased proportions of 20:4ω6 and 22:5ω6. Feeding a partially hydrogenated EFA-deficient diet (SRG) resulted in a microsomal fatty acid profile similar to that observed by feeding the unhydrogenated EFA-deficient diet (OPS).

Feeding an EFA-deficient diet led to a significant increase in the proportion of 18:1 and a significant decrease in the proportions of 18:0, 18:2ω6, 18:3ω6, 20:3ω9, 20:4ω6 and 22:5ω6 in mitochondria (Table 5). Feeding HSBO resulted in an increase in 18:1 and 20:5ω3 and a decrease in 20:3ω9, and 20:4ω6 and 22:5ω6. Chicks fed SRG had mitochondrial fatty acid profiles similar to those fed OPS.

Both linoleic acid and arachidonic acid were decreased in cytosol (Table 6) by feeding the EFA-deficient diet, and 18:3ω3 and 20:5ω3 were increased by feeding HSBO. No differences were seen in the cytosolic fatty acid profiles of chicks fed SRG and OPS.

DISCUSSION

The only overt sign of EFA deficiency in the chicks of this experiment after three weeks of experimental feeding was a significantly lower mean body weight in chicks fed OPS and SRG compared to that of chicks fed HSBO and CO. Chicks fed OPS had a poorer feed conversion than those fed other dietary lipids. This may be due to the highly saturated nature of the lipid source, as well as to the low levels of linoleic acid in the diet. Earlier investigators have reported that fatty acids from saturated fats such as lard and tallow are poorly absorbed by young growing chicks and turkey poults compared to fatty acids from vegetable oils (19,20).

The failure of chicks to develop severe EFA deficiency symptoms after 3 wk of feeding low linoleate diets is not surprising. Holman (21), in his review of EFA deficiency, discusses early studies in which day-old chicks grew normally for 6–8 wk on fat-free diets. Biochemical changes would be expected earlier, however, and changes in fatty acid profiles were observed herein.

EFA deficiency in rats and chicks is classically accompanied by decreased tissue levels of linoleic acid and arachidonic acid and by increased levels of de novo synthesized ω9-series fatty acids, oleic acid and its elongation product, 20:3ω9 (21,22). In the present work, feeding chicks the EFA-deficient OPS diet for 3 wk led to a significant reduction in 18:2ω6 and 20:4ω6 in all three liver subcellular fractions. Similarly, significant reductions in linoleic and arachidonic acids were seen in liver microsomes and mitochondria of chicks fed SRG. The lowered linoleic acid levels in cytosol were significant for SRG-fed chicks, but the arachidonic acid levels were not. The profiles of microsomal and mitochondrial lipids appeared to be more severely affected by feeding EFA-deficient diets than were the fatty acid profiles of cytosol lipids. Intermediates in the ω6 desaturation pathway were lowered in membrane lipids, as was the elongation product, 22:5ω6. The results from this experiment support the findings of Haeffner and Privett (23) that rats fed hydrogenated coconut oil (deficient in EFA) had decreased linoleic and arachidonic acid levels in mitochondrial phospholipids.

Increased levels of 20:3ω9 in tissue lipids have been used as a criteria for assessing EFA deficiency. This fatty acid is formed when the rate-limiting desaturation of 18:1ω9 is no longer inhibited by the presence of the more preferred substrates for the Δ6 desaturase, 18:3ω3 and 18:2ω6 (4). In the present experiment, however, levels of 20:3ω9 were depressed in chicks fed SRG, OPS and HSBO compared to the controls fed CO.

In rats, feeding certain hydrogenated fats can depress desaturation in the ω9 series. Thomassen et al. (1) reported that EFA deficiency in rats caused by feeding partially hydrogenated vegetable oil with virtually no linoleic acid was accompanied by elevated 20:3ω9 levels and a triene-to-tetraene ratio above 0.4. The elevated 20:3ω9 was less dramatic, however, when partially hydrogenated marine oils low in 18:2ω6 were fed. In a study by Hill et al. (10), the effect of EFA deficiency on triene-to-tetraene ratios also varied with dietary lipid source. Although levels of 20:3ω9 were elevated as a result of EFA deficiency, levels of 20:3ω9 were lower and precursor 20:2ω9 levels higher in liver phospholipids of rats fed partially hydrogenated soybean oil for 31 wk than in those fed hydrogenated coconut oil or low-fat corn oil diets. This effect was probably caused by impaired Δ5 desaturase activity by 18:1 isomers present in the partially hydrogenated soybean oil.

The source of dietary carbohydrate can also influence the response of experimental animals to the feeding of EFA-deficient diets. Trugnan et al. (24) reported that rats fed EFA-deficient diets containing hydrogenated coconut oil and sucrose developed the characteristic feature of EFA deficiency (reduced weight gain and decreased linoleic and arachidonic acid levels and increased 20:3ω9 levels in plasma phospholipids). When starch was fed in place of sucrose, however, the EFA deficiency symptoms were noticeably reduced.

Increased levels of 20:3ω9 in liver and heart phospholipids from EFA-deficient chicks fed diets containing isolated soy protein, corn starch and triolein for 3 wk have been demonstrated by Watkins (25). Possibly the types of dietary fat sources are responsible for the differing effects of EFA deficiency on ω9 desaturation in the present experiment. The fatty acid profile of each tissue is influenced in part by the dietary lipid source and by de novo synthesis of fatty acids. In addition, alteration of dietary fatty acids in liver microsomes by desaturation and elongation, rates of fatty acid oxidation and selective incorporation of certain fatty acids into specific lipid classes contribute to the tissue fatty acid profile (4).

A decrease in 20:4ω6 by isomers of 18:1 other than 18:1ω9 was reported in rats (11) and examined more closely by Lawson et al. (3) using concentrates of *cis* and *trans* isomers. *Trans* 18:1 isomers suppressed 20:4ω6 and 20:3ω9 levels and increased 18:2ω6 and 20:5ω3 levels in rat liver phosphatidylcholine and phosphatidylethanolamine. *Cis* 18:1 isomers suppressed 20:4ω6 levels in phosphatidylcholine, 20:5ω3 in phosphatidylcholine and phosphatidylethanolamine and 18:2ω6 in phosphatidylcholine, but increased levels of 20:4ω6 in phosphatidylethanolamine and 20:3ω9 in phosphatidylcholine and phosphatidylethanolamine. Mahfouz et al. (11) suggested that Δ6 desaturation of fatty acids was inhibited by 18:1 isomers in rats. Possibly the same mechanism is effective in chicks, since levels of 18:2ω6 were similar in liver fractions from chicks fed HSBO and CO, but levels of arachidonic acid were significantly lower in microsomes and mitochondria when HSBO was fed.

In the present experiment with chicks, feeding partially hydrogenated soybean oil also led to an increase in ω3 fatty acids. This increase may partially be due to the higher level of linolenic acid in the HSBO diet. (All other lipid sources in this experiment contained much lower levels of 18:3ω3).

In summary, the dietary lipids fed in this study had a significant effect on the fatty acid profiles of chick liver microsomes, mitochondria and cytosol. Feeding day-old chicks diets deficient in linoleate for 3 wk led to significant reductions in both linoleate and arachidonate. Feeding HSBO that had adequate levels of linoleate and linolenate also resulted in lowered arachidonate levels in liver fractions, suggesting that the 18:1 isomers present in the dietary lipid inhibited Δ6 desaturase activity. The effects of feeding a partially hydrogenated fat with low linoleate levels (SRG) were similar to the effects of feeding OPS. Thus, under the conditions of our experiment, the effects of feeding EFA-deficient diets and hydrogenated fats were not additive.

Further studies, to determine the effects of hydrogenated fats on linoleate metabolism after prolonged feeding in chickens are warranted. Such studies are important because blended fats containing *cis/trans* isomers of unsaturated fatty acids are used in poultry rations, and the expected accumulation of *cis/trans* isomers in poultry products would contribute to the total intake of these fatty acids in the diets of humans.

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Response of Urinary Malondialdehyde to Factors that Stimulate Lipid Peroxidation In Vivo

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Malondialdehyde (MDA) derivatives occur as normal constituents of rat and human urine. In a previous study, it was found that MDA excretion in rats is responsive to MDA intake and to certain factors that increase lipid peroxidation in vivo: vitamin E deficiency, iron administration and a high concentration of cod liver oil (CLO) fatty acids in the tissues. In the present study, the effect on MDA excretion of several additional dietary and endogenous factors was evaluated.

The composition of dietary fatty acids had a major influence on MDA excretion in fed animals, being highest for animals fed n-3 fatty acids (20:5 and 22:6) from CLO, intermediate for those fed n-6 (18:2) acids from corn oil (CO) and lowest for those fed saturated acids from hydrogenated coconut oil (HCO). Diet was the main source of urinary MDA in all groups. Fasting produced a marked increase in urinary MDA, which tended to be higher in rats previously fed CLO. Fasting MDA excretion was not affected by the level of CO in the diet (5, 10 or 15%), indicating that feeding n-6 acids does not increase lipid peroxidation in vivo. Adrenocorticotropic hormone and epinephrine administration increased urinary MDA, further indicating that lipolysis either releases fatty acid peroxides from the tissues or increases the susceptibility of mobilized fatty acids to peroxidation. A decrease in fasting MDA excretion was observed in rats previously fed a high level of antioxidants (vitamin E + BHT + vitamin C) vs a normal level of vitamin E. MDA excretion increased following adriamycin and CCl_4 administration. No increase was observed following short-term feeding of a choline-methionine–deficient diet, which has been reported to increase peroxidation of rat liver nuclear lipids.

This study provides further evidence that urinary MDA can serve as a useful indicator of lipid peroxidation in vivo when peroxidation of dietary lipids is precluded.

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Derivatives of malondialdehyde (MDA), a major carbonyl product of lipid peroxidation arising mainly from polyunsaturated fatty acids (PUFA), are normal constituents of rat and human urine. In a previous study (1), endogenous MDA excretion in the rat was found to increase in response to certain factors that enhance lipid peroxidation in vivo: vitamin E deficiency, iron nitrilotriacetate administration and a high PUFA intake from cod liver oil. In the present study, the effect of several additional factors on lipid peroxidation in vivo was evaluated using urinary MDA as an indicator: feeding saturated vs n-6 vs n-3 fatty acids; a high level of dietary antioxidants; administration of adrenocorticotropic hormone (ACTH), epinephrine (EP), adriamycin or CCl_4; and short-term choline-methionine deficiency.

METHODS

Experiment 1. The effect of feeding diets containing saturated vs n-6 (18:2) vs n-3 (20:5 and 22:6) fatty acids on lipid peroxidation in vivo was assessed in rats by the urinary MDA method. The composition of the diets is given elsewhere (1). The fat sources were as follows: 15% hydrogenated coconut oil (HCO); 5% corn oil (CO) + 10% HCO; 10% CO + 5% HCO; 15% CO; 5% CO + 5% HCO + 5% cod liver oil (CLO). All diets contained 30 IU vitamin E/kg as DL-α-tocopheryl acetate and were stored at 4 C. They were fed to five groups of eight male weanling Wistar rats (Woodlyn Laboratories, Guelph, Ontario). Body weights were determined weekly. After 10 wk, 24-hr urine samples were collected from fed rats and analyzed for total MDA by the high performance liquid chromatography (HPLC) method of Bird et al. (2) as modified for urine by Draper et al. (1). To determine the endogenous contribution to urinary MDA, the animals then were fed the PUFA-free 15% HCO diet for 48 hr, and urinary MDA was measured during the second 24-hr period. Finally, the animals were fasted for 48 hr, and MDA excretion was determined during the second 24 hr.

Experiment 2. After it was found in Experiment 1 that fasting increased MDA excretion, the effect of the lipolytic agents EP and ACTH on MDA excretion was investigated. These hormones were administered to groups of six adult male Wistar rats that had been fed a high-PUFA (10% CO + 5% CLO) diet for 4 wk. To eliminate the effect of dietary MDA on MDA excretion, the animals were fed a PUFA-free (15% HCO) diet for 48 hr, and basal MDA excretion was determined for the second 24 hr. Then they were injected intraperitoneally with saline (0.9%), ACTH (40 OU kg) or EP (1 mg/kg) and fed the 15% HCO diet for an additional 24 hr, during which time MDA excretion was again determined.

Blood samples were collected from the orbital sinus into tubes containing heparinized 0.9% saline (25 IU heparin/dl) prior to and 4 hr following administration of saline, ACTH or epinephrine. Samples also were collected from a group of six adult male rats (365 ± 10.5 g) fed Rodent Chow® prior to and following a 48-hr fast. Free fatty acids (FFA) in the plasma were determined by the method of Falholt et al. (3) as modified by Lee and coworkers (4).

Experiment 3. The effect of dietary antioxidants on urinary MDA was investigated. Diets containing n-6 (10% CO) or a mixture of n-6 and n-3 (10% CO + 5% CLO) fatty acids were fed for 4 and 6 wk, respectively, in conjunction with a mixture of antioxidants (300 IU DL-α-tocopheryl acetate/kg + 0.5% BHT + 0.5% vitamin C) or

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Abbreviations: MDA, malondialdehyde; PUFA, polyunsaturated fatty acids; ACTH, adrenocorticotropic hormone; EP, epinephrine; HCO, hydrogenated corn oil; CO, corn oil; CLO, cod liver oil; HPLC, high performance liquid chromatography; FFA, free fatty acids; CMD, choline-methionine-deficient; EFA, essential fatty acids.

a normal level of vitamin E (30 IU DL-α-tocopheryl acetate/kg). Fat and antioxidant additions were made at the expense of cornstarch (1). Final 24-hr urine samples were collected in the fed state for MDA analysis. The 10% CO groups then were fasted for 48 hr, and endogenous MDA excretion during the final 24 hr was measured. The 10% CO + 5% CLO groups were fed a PUFA-free (15% HCO) diet for 48 hr, and MDA excretion was determined during the final 24 hr. They then were fasted for 48 hr, and fasting MDA excretion during the final 24 hr was again determined.

Experiment 4. The toxicity of CCl_4 has been attributed to peroxidation of hepatic lipids. Its effect on urinary MDA was investigated in two groups of eight adult male Wistar rats (~300 g) fed a PUFA-free (15% HCO) diet. MDA excretion during the 24 hr before treatment and during two 24-hr periods following intubation with CCl_4 (100 μl/100 g) or saline (0.9%) was determined. MDA excretion also was assessed in saline-treated rats given 8.0 g food (the amount consumed by the CCl_4-treated animals 0–24 hr following intubation) to test the possibility that the response to CCl_4 was affected by a depressed intake of food relative to that of the controls (21.0 g).

Experiment 5. Adriamycin (doxorubicin), an antitumor antibiotic (5,6), causes cardiac toxicity associated with lipid peroxidation (7). Experiment 5 was designed to determine whether this effect could be detected in terms of increased MDA excretion. Two groups of eight male Wistar rats (~425 g) were fed for 3 days a 5% HCO diet (Dyets Inc., Bethlehem, PA) containing no detectable MDA (<0.1 μg/g). On day 4, the animals were injected intraperitoneally with either saline (0.9%) or adriamycin hydrochloride (5 mg/kg) in saline. Food consumption and MDA excretion were monitored for 48 hr post-administration.

Experiment 6. Based on the appearance of diene conjugates absorbing at 232 nm in the liver nuclei of rats fed a choline-methionine–deficient (CMD) diet, Rushmore et al. (8) proposed that lipid peroxidation was involved in the subsequent development of a high incidence of hepatic tumors. Through the courtesy of Dr. A. K. Ghoshal (Dept. of Pathology, University of Toronto, Ontario), urine samples from CMD animals collected at the peak concentration of diene conjugates (48–72 hr) and from controls were obtained for MDA analysis.

Statistical analysis. The significance of differences in weight, food consumption and MDA excretion was determined using Student's t-test, except that in Experiment 1, differences in MDA excretion were evaluated using Duncan's multiple-range test. Significance was tested at the 5% and 1% levels of probability.

RESULTS AND DISCUSSION

PUFA intake and MDA excretion. Figure 1 illustrates the effects of feeding diets high in saturated, n-6 and n-3 fatty acids on MDA excretion. The composition of dietary fatty acids had a major influence on MDA excretion in fed rats, being highest for those fed n-3 acids from CLO, intermediate for those fed n-6 acids from CO and lowest for those fed saturated acids from HCO. There were no significant differences ($p > 0.05$) in MDA excretion between groups fed different levels of corn oil (5, 10 or 15%). Switching the CLO and CO groups to the PUFA-free 15% HCO diet resulted in a major decrease ($p < 0.01$) in MDA excretion and eliminated treatment differences. This observation indicates that, as reported previously (1), diet was the main source of urinary MDA in these groups. The rats fed the essential fatty acid (EFA)-deficient 15% HCO diet for 10 wk had a significantly ($p < 0.01$) lower body weight than the other groups (data not shown).

A 24-48 hr fast produced a marked increase ($p < 0.01$) in MDA excretion in all groups. This increase was highest in the rats previously fed CLO, but the difference was significant ($p < 0.01$) only with reference to those fed 15% CO or 15% HCO. A significant increase in fasting MDA excretion in animals fed a diet containing cod liver oil was seen in an earlier study (1). These increases presumably reflect an enhanced peroxidation of tissue lipids. Reddy et al. (9) observed that the adipose tissue of animals fed CLO had three times the fluorescence of tissue from animals fed corn oil. Iritani et al. (10) found that chemiluminescence emission associated with NADPH-dependent lipid peroxidation in liver microsomes was greater in rats fed 5% vs 0.5% CO but not 10% vs 5% CO.

There were no significant differences in fasting MDA excretion among groups fed different levels of corn oil. Lack of a dose response to CO intake may be due to the fact that the conversion of linoleate to arachidonic acid (the presumed main source of MDA from peroxidation of n-6 acids) does not increase in proportion to the level of linoleate in the diet. The more peroxidizable pentaenoic and hexaenoic acids of the n-3 series, on the other hand, are readily deposited in the tissues of animals fed CLO, though not extensively in adipose tissue (1). The results indicate that high intakes of oils containing dienoic n-6 acids do not enhance peroxidation of PUFA in vivo.

It is noteworthy that urinary MDA also increased in fasting rats fed the HCO diet. This increase, which was comparable to that of rats fed the CO diets, may have been due to peroxidation of 5,8,11-eicosatrienoic acid, which accumulates in plasma and tissue phospholipids in EFA deficiency.

Effect of ACTH and EP. After 4 wk, MDA excretion on the high-PUFA diet averaged 3.1–3.6 μg/24 hr ($p > 0.05$) (Table 1). These values decreased to 1.1–1.3 μg/24 hr on the PUFA-free diet. Small but significant ($p < 0.05$) increases in MDA excretion over that of the control group were observed during the 24 hr following ACTH or EP administration.

Plasma FFA levels were 36.5% and 57.7% above control values 4 hr following EP and ACTH administration, respectively (Table 2). In animals fasted for 48 hr, FFA levels increased by 78.0% (362 ± 4.8 vs 203 ± 2.2 μmol/dl). The smaller increments in MDA excretion observed

TABLE 1

Effect of ACTH and Epinephrine (EP) Administration on Urinary MDA*

	Preinjection	Postinjection
Saline (0.9%)	1.2 ± 0.2^a	2.0 ± 0.2^a
ACTH (40 U/kg)	1.3 ± 0.3^a	3.7 ± 0.3^b
EP (1 mg/kg)	1.1 ± 0.2^a	3.6 ± 0.2^b

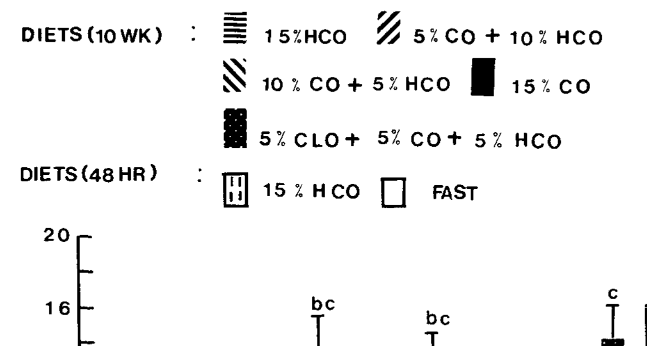
*μg/24hr, mean ± SEM, n = 6. Column means with different superscripts are significantly different ($p < 0.01$).

TABLE 2

Plasma Free Fatty Acid (FFA) Levels Following Andrenocorticotropic Hormone (ACTH) and Epinephrine (EP) Administration*

Treatment	FFA (μmol/dl)		
	Preinjection	Postinjection	% Change
Saline (0.9%)	160.0 ± 1.5[a]	164.0 ± 1.7[a]	2.5 ± 0.2[a]
EP (1 mg/kg)	145.0 ± 2.6[a]	198.0 ± 3.1[b]	36.5 ± 1.2[b]
ACTH (40 U/kg)	168.0 ± 3.0[a]	265.0 ± 4.1[c]	57.7 ± 2.1[c]

*Mean ± SEM, n = 6. Column means with different superscripts are significantly different ($p < 0.01$).



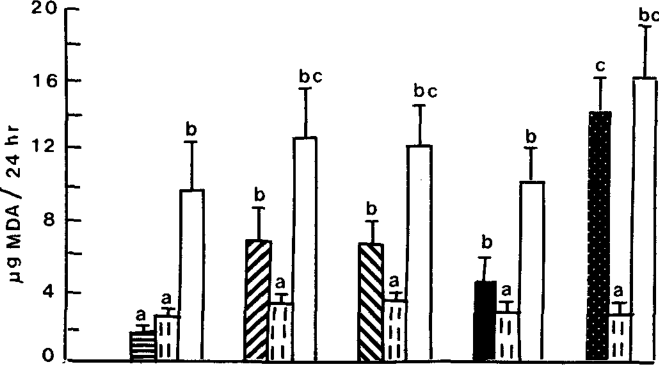


FIG. 1. **MDA excretion by rats fed different sources of fat. Mean ± SEM, n = 8. Bars with different letters are significantly different ($p < 0.01$).**

following ACTH or EP administration than those observed after fasting (Fig. 1) may have been due to a lesser lipolytic response.

The increase in MDA excretion following fasting or administration of lipolytic hormones may reflect a mobilization of fatty acid peroxides from the tissues. Alternatively, it is possible that mobilized PUFA are subject to peroxidation catalyzed by heme proteins or transition metals during transport in the serum. This question is worthy of further investigation.

Antioxidant effect. Feeding a high level of antioxidants was associated with reductions of 37% and 32% in fasting MDA excretion relative to that seen in controls fed a moderate level of vitamin E ($p < 0.01$) (Table 3). As the urine samples were collected 24–48 hr after the beginning of a fast, these reductions presumably reflect an inhibition of in vivo lipid peroxidation. This conclusion is supported by the fact that the reduction persisted in fasted animals after interim feeding of a peroxide-free diet. A similar effect was seen previously in fasted rats fed 100 IU or 30 IU vitamin E/Kg diet vs no antioxidant (1). Although the absolute decrease in MDA excretion associated with the high antioxidant intake was small, urinary MDA probably represents only a small fraction of the total MDA formed in vivo. ^{14}C-MDA administered orally to rats is extensively oxidized to $^{14}CO_2$, with only 10% of the radioactivity appearing in the urine (11). Ongoing experiments have indicated that urinary MDA consists mainly of lysine derivatives (unpublished results).

As observed previously (1), CLO markedly increased MDA excretion, an effect attributable mainly to enhanced peroxidation of dietary lipids (α-tocopherol was present as the acetate). Body weight gain was reduced in the high antioxidant group, probably as a result of the presence of BHT, which is known to depress growth at high concentrations. The elevation in urinary MDA associated with fasting was again evident in this experiment.

CCl_4 experiment. MDA excretion increased ($p < 0.01$) during the 24 hr following CCl_4 administration (Table 4), reflecting a stimulatory effect on in vivo lipid peroxidation. However, there was also a significant decrease in food intake by the CCl_4-treated rats (8.0 ± 0.9 g vs 21.0 ± 2.6 g) during the same period, raising the possiblity that the increase was due to inanition. Restricting the control rats to 8.0 g diet (which was eaten early in the 24-hr period) raised their MDA excretion to 2.4 ± 0.2 μg/24 hr ($p < 0.01$). This increase was significantly lower

TABLE 3

Effect of Dietary Antioxidants on MDA Excretion*

Group	10% CO		10% CO + 5% CLO		
Status Time	Fed 6 wk	Fasted 48 hr	Fed 4 wk	Fed 15% HCO 48 hr	Fasted 48 hr
Low antioxidant†	2.1 ± 0.3[a]	4.1 ± 0.4[a]	6.4 ± 0.5[a]	2.7 ± 0.2[a]	5.6 ± 0.6[a]
High antioxidant**	2.1 ± 0.2[a]	2.6 ± 0.2[b]	6.1 ± 0.4[a]	2.6 ± 0.3[a]	3.8 ± 0.2[b]

*Final 24 hr of each time period (μg); n = 8. Column means with different superscripts are significantly different ($p < 0.01$).
†30 IU DL-α-tocopheryl acetate/kg diet.
**300 IU DL-α-tocopheryl acetate + 0.5% BHT + 0.5% vitamin C/kg diet.

TABLE 4

Stimulation of Urinary MDA by CCl_4 Intubation*

Treatment†	CCl_4	Saline
Before dose (24 hr)	1.4 ± 0.2[a]	1.3 ± 0.3[a]
After dose (0–24 hr)	3.8 ± 0.2[a]	1.6 ± 0.15[b]
After dose (24–48 hr)	3.0 ± 0.25[a]	2.0 ± 0.2[a]

*µg MDA/24 hr, mean ± SEM, n = 8. Row means with different superscripts are significantly different ($p < 0.01$).
†100 µl/100 g; 15% HCO diet.

($p < 0.01$) than that of the rats intubated with CCl_4 (3.8 ± 0.2 µg/24 hr). Hence, the increase in MDA excretion following the CCl_4 dose does not appear to have been due entirely to lipolysis arising from a coincident decrease in food consumption. Failure to observe an effect of CCl_4 on urinary MDA in a previous study (1) was probably due to a decrease in MDA intake from the diet (Rodent Chow®) by the CCl_4-treated animals. Thus, tests for lipid peroxidation in vivo using MDA excretion as a criterion should be carried out using an MDA-free diet.

Adriamycin experiment. A small but significant ($p < 0.05$) increase in urinary MDA was observed from 0 to 24 hr, but not from 24 to 48 hr, following adriamycin administration (Table 5). There was also a small reduction in food consumption following its administration (17.5 ± 1.2 g vs 15.9 ± 1.4 g). The tenuous increase in urinary MDA may be due to the organ specificity of this compound.

Effect of choline-methionine deficiency. There was no significant change in MDA excretion caused by short-term feeding of a CMD diet (4.0 ± 0.5 µg/24 hr in the CMD group vs 3.5 ± 0.8 µg/24 hr in controls). Lipid peroxidation caused by short-term feeding of the CMD diet is reportedly confined to hepatic nuclei (8); any increase in MDA production at this site, therefore, may have a negligible effect on total MDA excretion.

TABLE 5

Effect of Adriamycin on Urinary MDA*

Treatment†	Time after administration	
	0–24 hr	24–48 hr
Saline (0.9%)	0.9 ± 0.1[a]	1.7 ± 0.3[a]
Adriamycin (5 mg/kg)	1.5 ± 0.1[b]	1.7 ± 0.4[a]

*µg MDA, mean ± SEM, n = 8. Column means with different superscripts are significantly different ($p < 0.05$).
†Animals fed PUFA-free 15% HCO diet.

Forms of MDA in urine. Anion exchange chromatography of rat and human urine has been used to separate several discrete MDA derivatives. In addition to N-acetyl-ε-propenal lysine (12), a cyclized form of ε-propenal lysine, enaminals of the phospholipid bases serine and ethanolamine and, in some samples, a small amount of free MDA have been identified (Hadley, M., and Draper, H.H., unpublished results). Together, these compounds represent a large fraction of the total MDA in most urine samples. The identity of several minor MDA-yielding compounds is under investigation, including the possibility that there may be lipids in urine from which MDA is formed as an artifact during the procedure described for its estimation.

General conclusion. The results of this study provide further evidence that urinary MDA is useful as an indicator of in vivo lipid peroxidation when peroxidation of dietary lipids is precluded.

ACKNOWLEDGMENT

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Effects of Diets Enriched in Eicosapentaenoic or Docosahexaenoic Acids on Prostanoid Metabolism in the Rat

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To clarify the effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) on prostaglandin biosynthesis, diets supplemented with oils rich in one fatty acid or the other were fed to rats over a 4-wk period. Animals fed the Max EPA diet showed a significant decrease in plasma and tissue phospholipid arachidonic acid content. While plasma levels of DHA increased on a shark liver oil diet enriched in DHA, the liver and kidney phospholipid contents of DHA were not altered. In addiition, the DHA-enriched diet did not decrease the arachidonic acid content of either liver or kidney phospholipids. Whole blood thromboxane and vascular prostacyclin synthesis were decreased by 65% and 36%, respectively, in animals fed the Max EPA diet. No such decrease was seen in the rats fed DHA-enriched diets. We conclude from these results that in the rat DHA is not likely to have a significant effect on prostaglandin synthesis when given as a dietary supplement.
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Dietary fish oils are generally thought to have a cardiovascular protective effect, which is attributed mainly to their ability to modify prostaglandin and lipid synthesis (1–4). While fish oils invariably contain a mixture of fatty acids, including eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3), most biological activity has been ascribed to EPA. For example, dietary EPA can depress the production of thromboxane A_2 (TXA_2) by reducing platelet phospholipid arachidonic acid stores and by competitively inhibiting cyclooxygenase (5,6). However, at least one in vitro study has shown that DHA is also a strong inhibitor of prostaglandin biosynthesis (7). Von Schacky and Weber (8) supplemented the human diet separately with purified EPA and DHA, and although thromboxane formation was unaltered, DHA reduced platelet responsiveness, indicating that it may contribute to the antithrombotic effects of dietary fish oils.

We have carried out detailed studies on the effects of dietary fish oils on phospholipid fatty acid composition and prostaglandin synthesis in the rat (9,10) using commercially available cod liver oil and Max EPA, which are richer in EPA than in DHA. It is known that Australian fish species have a different fatty acid composition compared to Northern Hemisphere species commonly used in commercial oil production (11). A local fish liver oil was found to be highly enriched in DHA, with relatively small amounts of EPA and arachidonic acid, while the total ω3 fatty acid content was similar to Max EPA oil. To elucidate further the effectiveness of EPA and DHA as prostaglandin inhibitors, diets supplemented with oils enriched in one fatty acid or the other were fed to rats over a 4-wk period. Changes in plasma and tissue phospholipid fatty acid composition and prostanoid production were compared among Max EPA oil, a local shark liver oil and a control diet of coconut oil/3% safflower oil.

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Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2; RIA, radioimmunoassay.

MATERIALS AND METHODS

Animals and dietary treatment. Thirty male Sprague-Dawley rats (150–200 g) were randomly allocated to three groups of 10 and placed on a synthetic diet preparation. The synthetic diet consisted of (by weight) casein, 20%; macro-minerals, 2%; vitamin and trace mineral mix, 0.75%; cellulose, 7%; choline chloride, 0.25%; corn flour, 45%; sucrose, 15%; and fat, 10%. The dry components of the feed were prepared by Milne Stock Feed (Welshpool, Western Australia), and the oils were added in our laboratory before feed was baked into small biscuits. Fatty acid composition was not altered during the baking process, and fresh food was prepared each week. The fat component contributed 20% of the caloric content of the diet and was either Max EPA fish oil (R.P. Sherer Pty Ltd, Melbourne, Victoria, Australia), hydrogenated coconut oil containing 3% safflower oil, or a locally prepared shark liver oil. The major fatty acid composition of these oils (analyzed by gas chromatography) is outlined in Table 1. Body weights were measured weekly, and at the end of the 4-wk treatment period rats were placed in metabolic cages for 24-hr urine collection. Animals were anaesthetized with sodium pentobarbitone and killed. Approximately 10 ml of aortic blood was obtained together with samples of kidney and liver tissues and a strip of thoracic aorta.

Fatty acid analysis. Total lipids were extracted from plasma (100μl) with 1 ml of ether/methanol (5:1, v/v); 1% KCl solution (150 μl) was added to aid phase separation. Homogenized kidney and liver tissue were extracted with choloroform/methanol (2:1, v/v) and filtered. All extracts

TABLE 1

Fatty Acid Composition (%) of the Dietary Oils Used to Supplement the Synthetic Feed

Fatty acid	Coconut/safflower oil	Max EPA	Shark liver oil
14:0	73.0[a]	7.4	2.6
16:0	12.0	16.9	17.6
16:1	—	9.9	5.9
18:0	11.0	3.5	4.4
18:1	1.2	14.4	20.4
18:2	2.3	1.2	1.1
20:1	—	1.9	4.8
20:4	—	0.9	2.6
20:5	—	17.5	5.2
22:6	—	11.9	20.2

[a]Including < 14:0.

were evaporated to dryness under nitrogen. All extracting solvents contained butylated hydroxy toluene as antioxidant. Total phospholipids were isolated from crude lipid extracts by thin layer chromatography (Silica Gel 60 F_{254}, Merck, Darmstadt, FRG) using a solvent system of hexane/ether/acetic acid/ methanol (170:40:4:4, v/v/v/v) (9). Analysis of acylated fatty acids in total lipids and phospholipids was carried out by gas chromatography of their corresponding methyl esters. Methyl esters were prepared by treatment of extracts suspended in ether (200 μl) with one drop of tetramethyl ammonium hydroxide solution (20% in methanol) at room temperature for 30 min. After settling, the upper layer was transferred to another vial and evaporated under nitrogen. The residue was redissolved in cyclohexane and analyzed using a Hewlett Packard 5890 gas chromatograph fitted with a 25-m methyl silicone capillary column (0.2 mm id), Hewlett Packard type 19091A-102, with hydrogen carier gas at 80 kPa. The oven was temperature-programmed as follows: 217 C for 14.5 min, 7 C per min to 252 C, then 25 C per minute to 340 C with a final hold of 3.5 min. Peaks were identified by comparison to authentic standards, and peak areas were calculated automatically using a Packard 604 recording data processor.

Serum thromboxane B_2 (TXB_2) analysis. Following collection, blood samples were allowed to clot by incubation for 1 hr at 37 C. Serum was removed after centrifugation and stored at −20 C. Samples were diluted 1:500 in radioimmunoassay (RIA) buffer prior to direct analysis for TXB_2 as previously described (12). The validity and reliability of these assays have been established in detail (13).

Aortic prostacyclin synthesis. The thoracic portion of the aorta was cleared of adhering tissue and washed in ice-cold saline. One cm sections were cut into rings and incubated in Krebs-Henseleit buffer at 37 C for 30 min. The aortic segments were then removed, blotted dry and weighed. The incubates were stored at −20 C for analysis of the stable prostacyclin hydrolysis product 6-keto $PGF_{1\alpha}$ by RIA (12,13).

Urine prostaglandin analysis. The volume of 24-hr urine collections was recorded, and samples were stored frozen at −20 C. PGE_2 and 6-keto $PGF_{a\alpha}$ were assayed in urine samples by RIA after organic extraction and thin layer chromatography as previously described (9).

Statistics. Results are expressed as the mean and standard error of the mean (SEM). One-way analysis of variance was used to test for significant differences between all dietary groups. Differences between any two dietary groups were established by modified t statistics using Fischer's least significance test.

RESULTS

All animals had a satisfactory weight gain over the 4-wk treatment period. Final weights for each of the groups were as follows: hydrogenated coconut oil containing 3% safflower oil, 223 ± 8 g; Max EPA oil, 246 ± 5 g; and shark liver oil, 250 ± 8 g.

Plasma and tissue fatty composition. The fatty acid composition of total plasma lipids of rats fed for 4 wk on the synthetic diets containing 10% (by weight) oil supplements is given in Table 2. Animals fed the Max EPA diet showed 36% lower (P < 0.01) 20:4 levels compared to the coconut oil control group. This lower 20:4 level was accompanied by a level more than three-fold higher of 20:5 and a slightly higher 22:6 level in plasma lipids. The animals fed shark oil showed a higher (P < 0.01) 20:4 content in plasma lipids together with a slightly higher level of 22:6 (P < 0.01). The 20:5 content of plasma lipids did not differ from those of the control group.

TABLE 2

Fatty Acid Composition of Plasma Total Lipids

Fatty acid	HCO	EPA	SLO
16:0	26.5 ± 1.4[a]	25.9 ± 1.4	26.6 ± 1.6
16:1	3.2 ± 0.2	3.7 ± 0.2	2.7 ± 0.1
18:0	16.3 ± 0.6	12.1 ± 0.8*	13.1 ± 0.9*
18:1	26.8 ± 1.7	28.2 ± 2.6	28.0 ± 2.4
18:2	8.5 ± 1.0	7.6 ± 0.8	6.5 ± 0.4
20:4	10.8 ± 1.1	6.9 ± 1.0*	13.3 ± 1.3*
20:5	1.9 ± 0.4	6.4 ± 0.9**	2.0 ± 0.2
22:6	5.0 ± 0.8	6.3 ± 0.7	7.2 ± 0.7*

HCO, hydrogenated coconut oil + 3% safflower oil; EPA, Max EPA oil; SLO, shark liver oil.
[a]Mean ± SEM, percent of total fatty acids, n = 10 for each group.
* and **, Significant differences (P < 0.01 and P < 0.001, respectively) between the HCO control and other dietary groups.

Results of the fatty acid analysis of liver phospholipids after the 4-wk feeding period are shown in Table 3. The most pronounced changes occurred in the rats fed Max EPA whose levels of 20:4 were lower by 33% (P < 0.001) and of 20:5 were six-fold higher. In contrast, animals on the shark oil diet showed 28% higher 20:4 and no change in 20:5. These changes were associated with small decreases in 18:0, 18:2 and 22:6 in the shark oil group compared to the coconut oil group. The fatty acid composition of kidney phospholipids (Table 4) showed similar trends to those observed for liver phospholipids. In particular, the Max EPA group showed lower 20:4 and a substantially higher 20:5 compared to the coconut oil control. However, in contrast to the results obtained in the liver, the rats fed shark oil had slightly lower 20:4 and a modest rise in 20:5 (P < 0.001) in kidney phospholipids. Again, 22:6 levels were not significantly higher on the shark oil diet,

TABLE 3

Fatty Acid Composition of Liver Phospholipids

Fatty acid	HCO	EPA	SLO
16:0	19.7 ± 0.5[a]	24.1 ± 1.3*	20.0 ± 0.6
16:1	2.5 ± 0.1	2.8 ± 0.3	2.5 ± 0.2
18:0	24.9 ± 0.4	25.9 ± 1.3	23.2 ± 0.6
18:1	7.8 ± 0.6	8.8 ± 0.5	7.7 ± 0.3
18:2	8.4 ± 0.8	6.5 ± 0.5*	5.8 ± 0.2**
20:4	22.5 ± 0.6	15.0 ± 0.6**	28.9 ± 0.8**
20:5	1.2 ± 0.2	7.5 ± 0.6**	1.3 ± 0.2
22:6	13.2 ± 0.8	13.7 ± 1.0	11.7 ± 0.4

HCO, hydrogenated coconut oil + 3% safflower oil; EPA, Max EPA oil; SLO, shark liver oil.
[a]Mean ± SEM, percent of total fatty acids, n = 10 for each group.
* and **, Significant differences (P < 0.01 and P < 0.001, respectively) between the HCO control and other dietary groups.

TABLE 4

Fatty Acid Composition of Kidney Phospholipids

Fatty acid	HCO	EPA	SLO
16:0	19.1 ± 1.3[a]	20.2 ± 1.6	22.9 ± 1.3
16:1	1.7 ± 0.1	2.1 ± 0.1	1.9 ± 0.1
18:0	22.8 ± 0.8	22.3 ± 1.1	24.9 ± 1.2
18:1	12.9 ± 0.7	12.5 ± 0.5	13.3 ± 0.8
18:2	11.1 ± 1.0	7.7 ± 0.8*	6.1 ± 0.3**
20:4	27.2 ± 1.3	20.2 ± 0.9**	25.2 ± 1.2
20:5	—	7.9 ± 0.6**	2.0 ± 0.2**
22:6	3.0 ± 0.2	4.5 ± 0.3*	3.6 ± 0.4

HCO, hydrogenated coconut oil + 3% safflower oil; EPA, Max EPA oil; SLO, shark liver oil.

[a]Mean ± SEM, percent of total fatty acids, n = 10 for each group.

* and **, Significant differences ($P < 0.01$ and $P < 0.001$, respectively) between the HCO control and other dietary groups.

despite the large amounts of that fatty acid in shark liver oil. The 18:2 content of kidney phospholipids was lower on both of the fish oil diets compared to the coconut oil group ($P < 0.01$).

Prostaglandin synthesis. The whole blood production of TXB_2 (ng/ml serum) for each of the dietary groups is illustrated in Figure 1a. There was a 65% reduction ($P < 0.001$) in TXB_2 synthesis from animals fed the Max EPA diet compared to the coconut oil control group. The animals fed shark oil showed no significant difference in thromboxane production compared to the control group.

Prostacyclin production in incubated segments of aorta was assessed by measurement of the stable hydrolysis product 6-keto-$PGF_{1\alpha}$. The production of 6-keto-$PGF_{1\alpha}$ for each dietary group is illustrated in Figure 1b. These results show similar but less pronounced effects as those seen for serum thromboxane. The animals fed Max EPA were 36% lower ($P < 0.02$) in aortic prostacyclin production compared to the group fed coconut oil while production in the animals fed shark oil was not significantly different from controls.

There was no significant difference in urinary excretion of PGE_2 or 6-keto-$PGF_{1\alpha}$ among the dietary groups. The urinary excretions of PGE_2 and 6-keto-$PGF_{1\alpha}$, respectively, were as follows: coconut oil, 12.5 ± 1.8 and 14.1 ± 1.9 ng/24 hr; Max EPA, 12.1 ± 1.8 and 17.1 ± 1.6 ng/24 hr; shark oil, 12.4 ± 1.6 and 17.9 ± ng/24 hr. There was no significant differences in 24-hr urine volume among the dietary groups.

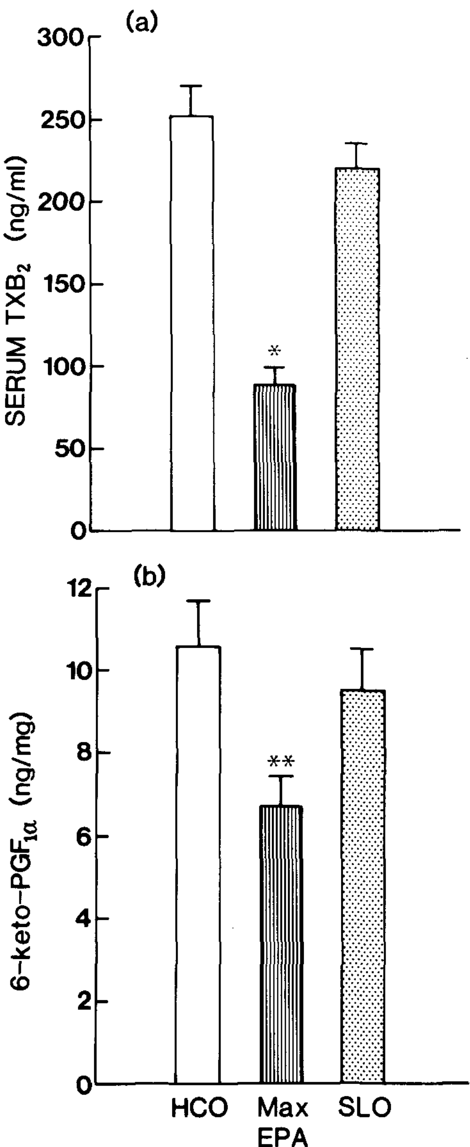

FIG. 1. The effects of dietary treatment with hydrogenated coconut oil containing 3% safflower oil (HCO), Max EPA fish oil (Max EPA) or shark liver oil (SLO) on (a) serum TXB_2 generation and (b) production of 6-keto-$PGF_{1\alpha}$ by incubated segments of aorta. *, $p < 0.001$ and **, $p < 0.02$ for differences between the HCO control groups and other dietary groups.

DISCUSSION

In this study, we have compared effects of dietary fish oils enriched with either EPA or DHA on tissue fatty acid composition and prostaglandin synthesis in the rat. We have found the dietary DHA is very poorly incorporated into tissue phospholipids and exerts a minimal influence on prostanoid synthesis.

The fatty acid composition of plasma lipids reflected the dietary fatty acid intake. Rats on the Max EPA diet showed higher levels of EPA and DHA and lower amounts of arachidonic acid. Those animals fed the shark oil diet showed a modest rise in DHA and arachidonic acid but no change in EPA compared to controls. Presumably the increased levels of arachidonic acid in plasma lipids are a result of the higher level (2.6%) of arachidonic acid in shark liver oil compared to Max EPA (< 1%) or the control diet.

In contrast to plasma lipids, DHA was not higher in either liver or kidney phospholipids from animals fed the shark oil diet. EPA was not increased in tissue phospholipids on this diet, while arachidonic acid was elevated in liver phospholipids but not in the kidney. These results are in contrast to other studies where rat platelet total phospholipids had a higher content of DHA when dietary DHA was increased from 0.8% to 1.6% of the total dietary

fatty acids (14). The lack of incorporation of DHA into tissue phospholipids is also in contrast to human studies where significant increases in platelet and leukocyte phospholipid DHA have been observed following diets enriched with DHA (8,15).

Rats fed the Max EPA diet showed a large rise in EPA content of both liver and renal phospholipids together with significantly lower arachidonic acid compared to the control group. These results are consistent with our previous studies (9,10) and those of others who fed fish oil diets to rats (16). The ability of EPA to be readily incorporated into tissue phospholipids at the expense of arachidonic acid is also indicated by its effectiveness at lowering prostanoid synthesis. Both whole blood thromboxane and vascular prostacyclin synthesis were lower in animals fed Max EPA. In previous experiments we have found that alterations in platelet phospholipid fatty acid composition after dietary Max EPA closely correlated with changes in plasma fatty acids and that these changes explain the observed effects on thromboxane production (10). No such decrease was seen in the rats fed shark oil which is not surprising in view of the lack of incorporation of DHA and EPA and the maintenance of arachidonic acid levels. The small but significant amount of arachidonic acid in the shark oil diet may compensate for the presence of EPA and DHA. This suggestion is supported by in vitro studies using human endothelial cells where DHA was incorporated into total phospholipids with a consequent reduction in arachidonic acid content; however, when cells were incubated with DHA and arachidonic acid together, the reduction in arachidonic acid was abolished and the uptake of DHA reduced (17).

Urinary excretion of PGE_2 and 6-keto-$PGF_{1\alpha}$ was not significantly different among the dietary groups. Although in previous experiments with large numbers of animals, 6-keto-$PGF_{1\alpha}$ excretion has been depressed by an EPA diet, results for PGE_2 have been less consistent (9,10). In man, urinary excretion of the major prostacyclin metabolites does not decrease on an EPA-enriched diet, although an increase in the 3-series prostanoids can be detected by gas chromatography–mass spectrometry (18). Our analytical procedure is unable to distinguish between the 2- and 3-series metabolites.

In vitro experiments using exogenous DHA indicate that this fatty acid is an inhibitor of prostanoid synthesis in ram seminal vesicles (7). This study shows, however, that in the rat DHA is not likely to have a significant effect on prostanoid synthesis when given as a dietary supplement due to its lack of incorporation into tissue phospholipids, particularly in the presence of small amounts of arachidonic acid. Even when DHA has been incorporated into platelet phospholipids, it is not released to any significant extent upon thrombin stimulation and can exert only minimal effects upon the arachidonic acid cascade (15). In a study similar to ours, Bruckner and coworkers (19) found that platelet DHA levels and serum TXB_2 were not altered in rats fed a DHA-enriched diet, while those fed an EPA-rich diet had significantly decreased TXB_2 levels. They conclude that EPA and not DHA is responsible for the alterations in platelet prostaglandin biosynthesis. Despite this, it cannot be ruled out that, at least in man, ingestion of DHA may influence platelet behavior by modifying plasma fatty acid composition (8).

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Lipid Composition of Cultured B16 Melanoma Cell Variants with Different Lung-colonizing Potential

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Lipid components influence several cell surface properties that are critical in different stages of the metastatic process. In this study, we examined whether the different lung-colonizing potential of B16-F1 and B16-F10 melanoma cells could be related to a characteristic lipid profile. The lipid analyses, carried out on the same cell cultures used for the assay of lung-colonizing potential, revealed characteristics in the lipid composition of both B16-F1 and B16-F10 melanoma cells that are common to other systems of malignant cells: a high level of 18:1 associated with low proportions of polyunsaturated fatty acids in phospholipids, accumulation of ether-linked lipids and absence of complex gangliosides. The two B16 melanoma variants differed significantly only with respect to ether-linked lipids, due to a higher level of alkyl-PC in B16-F10 than in B16-F1.
Lipids 22, 651–656 (1987).

The cell surface is known to be involved in the mechanism of metastatic diffusion of tumor cells (1). Indeed, it is through its cell surface that an invading malignant cell successfully interacts with a number of the host homeostatic mechanisms through several stages, ending with the occurrence of a secondary tumor at a distant site (2,3). Much information on biological and molecular changes of the cell surface of malignant cells has been accumulated over the last few years (4); data, however, are still lacking on which of these changes are relevant to the metastatic behavior of malignant cells. Those related to lipid structure (5,6) appear to be of particular importance. Specific lipid components might influence the metastatic potential of tumor cells through their effect on cell surface properties—permeability (7), adhesiveness (8), agglutinability (9), antigenicity (10), receptorial (11,12) or enzymatic (13) activity—that may be critical for metastastic diffusion.

The availability of tumor cell variants with different metastatic potential (14) has greatly improved our capacity to study the role of cell surface molecular characteristics in the metastatic process. From B16 melanoma, Fidler (15) selected B16-F1 cells, whose lung-colonizing potential is low, after one in vivo–in vitro cycle, and B16-F10 cells, with high lung-colonizing potential, after 10 in vivo–vitro cycles.

In the present study, we analyzed the various lipid components of F1 and F10 melanoma cells in order to investigate whether the different lung-colonizing potentials of the two variants may be correlated with a specific lipid pattern.

Some aspects of the lipid composition of F1 and F10 cells have recently been described by Schroeder and Gardiner (16), while Yogeeswaran et al. (17) and Raz et al. (18) have published data on their glycolipid structure.

MATERIALS AND METHODS

Cells and culture conditions. F1 and F10 variants of B16 melanoma were supplied by Dr. I. Fidler (Department of Cell Biology, University of Texas System Cancer Center, Houston). Upon arrival in our laboratory, the cells were cultured for a few passages and then frozen in liquid nitrogen.

For each experiment, cells were first thawed and then propagated for a few passages to obtain a sufficient number of cells to enable us to determine at the same time both their lung-colonizing potential and their various lipid components. Cells were seeded at 0.8×10^6 cells per dish (10-mm Falcon plastic culture dishes) and grown for 4 days at 37 C in antibiotic-free Eagle's minimal essential medium with a fourfold concentration of vitamins and essential amino acids (Flow Laboratories, Irvine, CA) supplemented with 10% fetal calf serum (Flow Labs) in a humidified atmosphere containing 5% CO_2. Cell cultures were passed at subconfluence by incubation at 37 C for 1 min with a 0.25% trypsin solution in phosphate-buffered saline (PBS). We monitored cultures periodically for mycoplasma contamination using Chen's fluorochrome test (19).

Determination of lung-colonizing potential. Cell cultures of F1 and F10 cells were harvested at the exponential growth phase by 1 min of trypsinization with 0.25% trypsin in PBS. Cells were washed by centrifugation followed by resuspension in PBS and then were counted by an automatic cell counter (Coulter Electronic, Luton, England). Only cells whose viability was greater than 95%, as demonstrated by Trypan blue exclusion test, were used. After adjusting cell concentration to 2.5×10^5/ml, 0.2 ml of single-cell suspensions was injected into the tail veins of six- to eight-week old C57B1/6 mice (Charles River Italia, Calco, Italy). Each assay was performed on 30 animals, which were killed 21 days after injection. Nodules on the lung surface were counted with the aid of a dissecting microscope. In three separate experiments, the mean number of lung nodules per animal inoculated with F1 and F10 cells was 3 (range 0–12) and 55 (range 15–136), respectively.

Lipid extraction and analysis. Cell cultures for lipid analyses were harvested by incubation at 37 C with a 0.5 mM EGTA solution in PBS. The EGTA-detached cells were washed twice by centrifugation followed by resuspension in PBS. Cells were counted and then sonicated with a Labsonic sonicator model 1510 (Braun, Melsungen, FRG). Total lipids were extracted from the sonicated cell

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Abbreviations: PBS, phosphate-buffered saline; TLC, thin layer chromatography; GLC, gas liquid chromatography; FAME, fatty acid methyl esters; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SP, sphingomyelin; PA, phosphatidic acid; PI, phosphatidylinositol; PS, phosphatidylserine; GL1, glucosyl-ceramide; GL2, lactosyl-ceramide; GL3, galactosyl-galactosyl-glucosyl-ceramide.

suspensions with chloroform/methanol (2:1, v/v) following the method of Folch et al. (20), and then were fractionated into neutral lipids and phospholipids by silicic acid column chromatography.

Neutral lipids were sequentially separated into cholesteryl esters, alkyldiacylglycerols, triacylglycerols, free fatty acids and free cholesterol by thin layer chromatography (TLC) on 0.25 mm Silica Gel H precoated plates (Merck, Darmstadt, FRG), using hexane/diethyl ether/acetic acid (85:15:1, v/v/v).

Cholesterol was determined in the total lipid extract and in the free and esterified cholesterol fractions by quantitative gas liquid chromatography (GLC) with coprostanol as an internal standard.

Triacylglycerols and free fatty acids were evaluated from quantitative GLC of their fatty acid methyl esters (FAME), which were obtained by acid-catalyzed methanolysis in the presence of an arachidic acid internal standard.

Alkyldiacylglycerols were converted into alkylglycerols by hydrogenolysis with sodium-bis(2-methoxy-ethoxy)aluminum hydride (Aldrich Chemical Co., Steinheim, FRG) in the presence of pentadecylglycerol as an internal standard (21) and were quantitatively assayed as isopropylidene derivatives by GLC.

Phospholipids in 1mg amounts were applied to 0.25 mm Silica Gel H precoated plates, activated at 110 C for 2 hr before use, and were chromatographed bi-dimensionally with chloroform/methanol/ammonia (95:45:11, v/v/v) followed by chloroform/methanol/acetic acid/water (90:40:12:2, v/v/v/v). Under these conditions, phospholipids were fractionated into diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SP) and phosphatidic acid (PA); phosphatidylinositol (PI) and phosphatidylserine (PS) were combined for analyses since they could not always be easily separated. After the plates were sprayed with 0.1% dichlorofluorescein in ethanol, the phospholipid fractions were localized under ultraviolet light and scraped into vials for phosphorus determination (22) after digestion with sulfuric acid/perchloric acid (3:2, v/v). PE and PC were submitted to ether-lipid analysis following Su and Schmid's procedure (21), in which the alkenyl-linked subfractions were converted into cyclic acetals in the presence of dimethoxytetradecane and the alkyl-linked subfractions were converted into alkylglycerols with pentadecylglycerol as its internal standard.

Glycolipids were determined radiometrically by measuring their radioactivity after exposing cells for 48 hr to growth media containing 1.0 μCi/ml of 3H-galactose (NEN Research Products, Dreieich, FRG). Neutral glycolipids and gangliosides were isolated from total lipids by Siakotos and Rouser's chromatographic procedure (23). Neutral glycolipids were then freed from the contaminating phospholipids and neutral lipids by acetylation followed by Florisil column chromatography according to Saito and Hakomori (24). Gangliosides and deacetylated neutral glycolipids were fractionated by TLC on high performance TLC plates (Merck) using chloroform/methanol/0.25% $CaCl_2$ (55:40:9, v/v/v) and chloroform/methanol/water (65:25:4, v/v/v), respectively. The labeled glycolipid fractions were revealed by fluorography and counted in scintillation vials. Glucosyl-ceramide (GL1), lactosyl-ceramide (GL2) and GM3 were identified by the carbohydrate group analysis, as we have previously described (25). GM2, GM1 and GD1a were identified by comparing their migration rates on TLC to those of authentic standards. The TLC of neutral glycolipids of F1 and F10 cells also revealed the presence of a more complex glycolipid, tentatively identified as galactosyl-galactosyl-glucosyl-ceramide (GL3) based on the similarity of its migration rate to that of an authentic standard. The oligosaccharide chain was not chemically characterized.

Gas chromatographic analyses. FAME were prepared from triacylglycerols, free fatty acids, cholesteryl esters and phospholipid classes by heating the sample at 80 C for 12 hr in a solution of 5% H_2SO_4 in methanol/benzene (25:1, v/v). FAME were extracted into hexane, concentrated under a stream of nitrogen and then analyzed by GLC in a Perkin Elmer Gas Chromatograph model 3920 equipped with hydrogen flame detectors. FAME were analyzed isothermally at 185 C on 6-ft glass columns packed with 10% EGSS-X on Gas-Chrom P 100–120 mesh (Supelco, Bellefonte, PA). FAME were identified by their retention times relative to those of standard methyl ester mixtures (Supelco).

The cyclic acetal derivatives of alkenyl groups and isopropylidene derivatives (26) of alkylglycerols were quantitatively analyzed at 195 C on 6-ft glass columns packed with 10% EGSS-X on Gas Chrom P 100/120 mesh.

Cholesterol was measured at 265 C on 6-ft glass columns packed with 3% OV 17 on Supelcoport 80/100 mesh (Supelco).

The methylglycoside products of the acid-catalyzed methanolysis of the glycolipids were analyzed as trimethylsilyl derivatives on 6-ft glass columns packed with 3% OV-17 on Supelcoport 80/100 mesh (Supelco) at a programmed temperature ranging from 120 C to 230 C (2 C/min).

Peak areas were measured with a Perkin-Elmer Sigma 10 integrator.

Statistical tests. Student's t-test was used to assess the statistical significance of the differences between F1 and F10 cells.

Lipid standards. We synthesized the standard of pentadecyl-glycerol (27). Dimethoxytetradecane, coprostanol and arachidic acid were supplied by Supelco.

RESULTS AND DISCUSSION

As shown in Table 1, the low metastatic F1 and the highly metastatic F10 melanoma cells contained comparable concentrations of total phospholipids and cholesterol, esterified cholesterol, triacylglycerols, alkyldiacylglycerols and free fatty acids. Schroeder and Gardiner, instead, reported a lower level of cholesterol in F10 than in F1 cells (16).

The two variants of B16 melanoma did not differ in their phospholipid composition (Table 2).

In both F1 and F10 cells, an appreciable proportion of PE was composed of alkenyl-linked molecules, while small percentages of alkyl-linked molecules were associated with PC (Table 3). This pattern is reminiscent of that found in different types of normal and malignant cells (29–41). Under our experimental conditions, alkyl-linked PE and alkenyl-linked PC were not detectable in

TABLE 1

Lipid Composition of B16-F1 and B16-F10 Melanoma Cell Lines[a]

Lipid Class	B16-F1	B16-F10
Total phospholipids[b]	49.6 ± 1.7	45.4 ± 2.4
Total cholesterol	6.8 ± 0.4	7.1 ± 0.7
Esterified cholesterol[c]	0.2 ± 0.1	0.2 ± 0.1
Triacylglycerols[d]	1.0 ± 0.1	1.1 ± 0.4
Alkyldiacylglycerols[e]	0.5 ± 0.2	0.6 ± 0.1
Free fatty acids	0.4 ± 0	0.4 ± 0

[a]Values, expressed as μg/10^{6}cells, are means ± SE of three separate experiments.

[b]As calculated by multiplying by 25 lipid phosphorus assayed on total lipids (22) after digestion with sulfuric acid/perchloric acid (3:2, v/v).

[c]Calculated from the gas chromatographic data of cholesteryl ester and free cholesterol thin layer chromatographic fractions and taking into account the value of total cholesterol in the total lipid extracts.

[d]Triacylglycerols (TG) were evaluated from the quantitative gas liquid chromatographic analysis of their fatty acid methyl esters using the following formula: (mol TG fatty acids/3) × molecular weight of TG. TG molecular weight was calculated on the basis of the fatty acid composition.

[e]Evaluated by the quantitative gas liquid chromatographic analysis of the isopropylidene derivatives of alkylglycerols using a 850 molecular weight as determined in the basis of the alkyl and acyl group composition.

TABLE 2

Phospholipid Composition of B16-F1 and B16-F10 Melanoma Cell Lines[a]

Phospholipid class	B16-F1	B16-F10
Diphosphatidylglycerol	5.1 ± 0.5	4.9 ± 0.4
Phosphatidylethanolamine	22.2 ± 0.8	23.4 ± 0.5
Phosphatidylcholine	54.9 ± 0.7	55.6 ± 1.6
Phosphatidylinositol + phosphatidylserine	11.3 ± 0.1	10.2 ± 1.0
Sphingomyelin	3.8 ± 0.2	3.9 ± 0.9
Phosphatidic acid	2.2 ± 0.1	1.7 ± 0.1

[a]Values, expressed as percentages of total phospholipid phosphorus (22), are means ± SE of three separate experiments.

either cell lines. The combined content of ether-linked lipids in F1 and F10 cells, calculated as a percentage of total lipids, yields values which are the lowest for the in vitro cell systems examined to date (39–41). Comparison of the ether-linked phospholipids of the two B16 melanoma variants revealed that F10 cells have a higher level of alkyl-PC than F1 cells, a difference analogous to that found by Friedberg et al. (42) between high- and low-metastatic mammary carcinomas.

Neutral glycolipids of F1 and F10 cells were composed primarily of glucosyl-ceramide and to a lesser extent of lactosyl-ceramide and of a fraction with a migration rate on TLC corresponding to that of a trihexosyl-ceramide standard (Table 4). GM3 accounts for 95% of total gangliosides of both F1 and F10 cells, the remainder being represented by GM2, GM1 and GD1a. GM3 was

TABLE 3

Percentages of Ether-linked Subfractions of Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC) from B16-F1 and B16-F10 Melanona Cell Lines

	Percentage of total phospholipid class[a]	
Subfraction	B16-F1	B16-F10
Diacyl PE	87.0 ± 6.1	87.2 ± 4.9
Alkenyl-linked PE	13.0 ± 0.8	12.8 ± 1.3
Alkyl-linked PE	N.D.[b]	N.D.[b]
Diacyl PC	97.5 ± 8.9	95.3 ± 9.8
Alkenyl-linked PC	N.D.[b]	N.D.[b]
Alkyl-linked PC	2.5 ± 0.4	4.7 ± 0.6[c]

[a]Percentages of the various subfractions were calculated as previously described (41). Values are means ± SE of five separate experiments.

[b]N.D., not detectable under the analytical conditions used.

[c]Significantly different from B16-F1 at $P < 0.02$.

the only ganglioside in the study by Yogeeswaran et al. (17), while Raz et al. (18) found minor amounts of GM2 and GM1 together with GM3 in these cell lines. These discrepancies may be related to differences, even in the same transformed cells, in the expression of the block in ganglioside biosynthesis (11). The proportion of the various neutral glycolipids and gangliosides did not differ significantly between the two variants of melanoma cells.

The fatty acid compositions of the individual lipid classes from F1 and F10 cells are reported in Tables 5–7. In both cell lines, the fatty acid profile of CE and of TG was characterized by a high proportion of 18:1 followed by 16:0, while FFA were composed mainly of 16:0 followed by 18:1 (Table 5). F10 cells showed a higher level of 18:0 in TG and FFA than F1 cells.

In both F1 and F10 cells, the overall pattern of fatty acid profiles of the individual glycerophospholipids (Table 6) and of sphingomyelin (Table 7) was similar to that found in other types of cells grown in tissue culture (39,43–45). Moreover, the various glycerophospholipids of F1 and F10 cells showed elevated proportions of 18:1 and very low levels of 20:4, 22:5 and 22:6 fatty acids, a characteristic common to several systems of malignant cells (36,43–51). On the whole, the fatty acid compositions of the various glycerophospholipids and of sphingomyelin did not differ significantly for the two cell lines. This observation is at variance with Schroeder and Gardiner's finding of a lower content of C20 and C22 polyunsaturated fatty acids in the phospholipids of F10 than in those of F1 cells (16).

The alkyl chain composition of alkyl-linked PC was characterized in both F1 and F10 cells by a high proportion of 16:0 followed by 18:1, 18:0 and 16:1 (Table 8), a pattern comparable to that found in L-M cells (39) and in untransformed and SV40-transformed Balb/c 3T3 cells (41). The alkenyl chain profile of alkenyl-linked PE was similar to that of PC alkyl chains, although this latter had a lower level of 16:0. There were no significant differences in the alkenyl and alkyl chain compositions between F1 and F10 cells.

The following conclusions can be drawn from the results of the present investigation: (a) both F1 and F10 cells

TABLE 4

Neutral Glycolipid and Ganglioside Composition of B16-F1 and B16-F10 Melanoma Cell Lines[a]

	Neutral glycolipids[b]			Gangliosides[c]			
Cell line	GL1	GL2	GL3	GM3	GM2	GM1	GD1a
B16-F1	77.1 ± 8.6	15.2 ± 4.8	7.6 ± 4.0	95.1 ± 0.4	1.6 ± 0.1	1.5 ± 0.5	1.9 ± 0.8
B16-F10	87.7 ± 1.6	8.8 ± 0.9	3.3 ± 1.1	96.0 ± 1.3	0.9 ± 0.2	0.7 ± 0.3	2.3 ± 0.9

[a]Values are percentages of the total neutral glycolipid or ganglioside-associated radioactivity. "Total" refers to the sum of the radioactivities of individual glycolipids fractionated by thin layer chromatography. Each value represents the mean ± SE of three separate experiments.

[b]GL1, glucosyl-ceramide; GL2, lactosyl-ceramide; GL3, galactosyl-galactosyl-glucosyl-ceramide.

[c]Gangliosides are indicated according to the nomenclature of Svennerholm (28).

TABLE 5

Fatty Acid Composition of Cholesteryl Esters, Triacylglycerols and Free Fatty Acids from B16-F1 and B16-F10 Melanoma Cell Lines[a]

	Cholesteryl esters		Triacylglycerols		Free fatty acids	
Fatty acid	B16-F1	B16-F10	B16-F1	B16-F10	B16-F1	B16-F10
14:0	10.6 ± 0.4	11.2 ± 0.8	4.9 ± 1.5	6.6 ± 0.4	5.0 ± 0.4	5.6 ± 0.3
16:0	28.2 ± 2.1	23.4 ± 3.2	36.5 ± 2.1	31.3 ± 1.9	43.7 ± 1.1	39.2 ± 1.7
16:1	8.0 ± 1.2	11.6 ± 1.2	6.2 ± 0.7	6.4 ± 0.4	4.0 ± 1.5	5.5 ± 0.6
18:0	11.6 ± 3.0	7.3 ± 0.9	9.0 ± 0.7	5.9 ± 0.4[d]	21.5 ± 0.7	16.0 ± 1.7[d]
18:1	40.0 ± 5.5	42.6 ± 3.0	41.2 ± 2.6	47.1 ± 1.2	17.0 ± 1.5	26.0 ± 3.4
18:2	1.1 ± 0.7	1.9 ± 0.6	1.1 ± 1.0	2.1 ± 1.6	2.7 ± 2.5	4.0 ± 1.1
20:4	1.4 ± 0.2	1.6 ± 0.5	N.D.[b]	N.D.[b]	3.1 ± 1.6	1.7 ± 0.3
22:5	N.D.[b]	N.D.[b]	0.2 ± 0.1	0.2 ± 0.2	0.1± 0.1	0.1 ± 0.1
22:6	N.D.[b]	N.D.[b]	tr.[c]	0.1 ± 0.1	1.1 ± 0.5	1.1 ± 0.2

[a]Values, expressed as % of total fatty acids, are means ± SE of three separate experiments.

[b]N.D., not detectable under the analytical conditions used.

[c]tr, trace amount lower than 0.1%.

[d]Significantly different from B16-F1 at $P < 0.05$.

TABLE 6

Fatty Acid Composition of Glycerophospholipids from B16-F1 and B16-F10 Melanoma Cell Lines[a]

	Diphosphatidylglycerol		Phosphatidylcholine		Phosphatidylethanolamine		Phosphatidylinositol + phosphatidylserine	
Fatty acid	B16-F1	B16-F10	B16-F1	B16-F10	B16-F1	B16-F10	B16-F1	B16-F10
16:0	21.0 ± 5.5	19.8 ± 3.5	40.6 ± 5.8	41.3 ± 4.8	25.6 ± 5.3	26.2 ± 3.0	13.3 ± 2.4	10.4 ± 1.9
16:1	18.2 ± 2.6	18.8 ± 1.9	7.1 ± 0.5	8.2 ± 0.8	4.6 ± 0.5	4.9 ± 1.1	2.7 ± 0.7	3.1 ± 0.9
18:0	8.9 ± 2.6	8.2 ± 2.0	6.3 ± 0.8	4.3 ± 0.9	16.1 ± 3.1	16.4 ± 4.5	41.9 ± 5.9	40.7 ± 7.2
18:1	41.2 ± 6.5	40.9 ± 5.6	43.6 ± 5.5	44.4 ± 4.8	44.8 ± 5.1	46.6 ± 5.8	36.2 ± 7.1	38.3 ± 7.5
18:2	4.4 ± 1.4	5.8 ± 0.4	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.2	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.3
20:4	6.7 ± 2.4	6.6 ± 2.0	0.6 ± 0.1	0.4 ± 0.1	4.5 ± 1.3	3.1 ± 0.2	4.8 ± 1.7	5.7 ± 1.4
22:5	N.D.[b]	N.D.[b]	0.1 ± 0.1	0.1 ± 0.1	1.8 ± 1.3	1.0 ± 0.3	0.3 ± 0.2	0.3 ± 0.3
22:6	N.D.[b]	N.D.[b]	0.1 ± 0.1	0.2 ± 0.1	1.5 ± 0.7	0.9 ± 0.2	0.3 ± 0.1	0.3 ± 0.1

[a]Values, expressed as % of total fatty acids, are means ± SE of three separate experiments.

[b]N.D., not detectable under the analytical conditions used.

TABLE 7

Fatty Acid Composition of Sphingomyelin from B16-F1 and B16-F10 Melanoma Cell Lines[a]

Fatty acid	B16-F1	B16-F10
16:0	48.3 ± 6.0	50.0 ± 2.4
16:1	0.3 ± 0.3	0.4 ± 0.3
18:0	22.2 ± 1.0	23.4 ± 2.6
18:1	5.5 ± 2.8	6.0 ± 2.8
22:0	5.0 ± 1.2	4.6 ± 1.0
22:1	3.2 ± 0.5	2.7 ± 0.1
23:0	1.7 ± 1.1	2.1 ± 0.6
23:1	N.D.[b]	N.D.[b]
24:0	7.8 ± 1.4	6.7 ± 1.9
24:1	7.6 ± 1.9	5.9 ± 0.3

[a]Values, expressed as % of total fatty acids, are means ± SE of three separate experiments.

[b]N.D., not detectable under the analytical conditions used.

showed characteristics in their phospholipid fatty acid compositions, ether-linked lipid contents and glycolipid patterns that are common to several systems of transformed cells; (b) despite the differences in their lung-colonizing potential, the two B16 melanoma variants did not differ in terms of their major lipid components and phospholipid fatty acid profiles, a result that raises the issue of whether previously reported differences (16) in the cholesterol content and in the polyunsaturated fatty acid level of the two B16 melanoma variants might not be attributable to biological factors other than metastatic potential; (c) the high alkyl-PC level in F10 cells, which has also been found in high-metastatic mammary carcinoma cells (42), is the only lipid characteristic that can be related to a high metastatic potential.

TABLE 8

Composition of Alkenyl and Alkyl Chains of Ether-linked Subfractions of Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC) from B16-F1 and B16-F10 Melanoma Cell Lines[a]

	Alkenyl-linked PE		Alkyl-linked PC	
Alkenyl or alkyl chain	B16-F1	B16-F10	B16-F1	B16-F10
16:0	48.6 ± 1.6	45.5 ± 2.4	58.1 ± 4.1	54.9 ± 2.8
16:1	5.3 ± 2.2	4.8 ± 1.0	3.6 ± 1.2	3.2 ± 1.3
18:0	12.4 ± 1.9	12.0 ± 2.2	8.9 ± 1.7	8.0 ± 3.0
18:1	33.7 ± 2.2	37.7 ± 2.2	29.4 ± 5.1	33.8 ± 4.8

[a]Values, expressed as mol % of total alkenyl and alkyl chains, are means ± SE of five separate experiments.

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[^{3}H]Cholesterol Transfer from Microemulsion Particles of Different Sizes to Human Fibroblasts

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A new technique for preparing microemulsion particles of well-defined sizes and compositions is presented. Utilization of these microemulsions is advocated as lipoprotein models in studies of lipid transport and metabolism, rather than the currently used phospholipid-cholesterol vesicles. The emulsion particles consisted of egg phosphatidylcholine and cholesterol as surface lipids and cholesteryl oleate as core lipid. They were prepared by a combined injection and sonication technique and size-separated by a two-step procedure of gel filtration chromatography and density gradient centrifugation. By varying the ratios of core and surface material, particles covering a size range of 20–200 nm in diameter could be produced. The adequacy of these microemulsions as lipoprotein models was tested by studying the transfer of [^{3}H]cholesterol and [^{14}C]cholesterol oleate from the particles to cultured human fibroblasts. Up to a particle size of 100 nm, there was a slight increase of [^{3}H]cholesterol transfer. The transfer of [^{14}C]cholesteryl oleate was very slow, yet measurable. Studies of the exchangeability of cholesterol between the microemulsion core and surface phases indicated that all cholesterol can be transferred from microemulsions to cultured cells as a single pool.
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Surface transfer of free cholesterol between lipoproteins or lipoprotein-like structures and cellular plasma membranes has been the subject of intensive studies for many years (for reviews, see 1 and 2). This transfer or exchange process can both deliver and remove cellular cholesterol (3), and it has been suggested that it could play an important role in the regulation of cellular cholesterol content (4,5). Investigations of the flux of free cholesterol between a large variety of acceptor and donor structures, including liposomes (6–8), membranes (9–11), lipoproteins (12–14) and protein-lipid complexes (15), have shown a rapid exchange of cholesterol between these structures.

When studying the specific case of surface transfer from lipoproteins to cellular membranes, the situation gets more complex as protein effects have to be eliminated to produce pure transfer data. Specific receptor-mediated cell uptake via the low density lipoprotein (LDL) receptor has been abolished by using receptor-negative cells (16–18) or by modifying the protein part of the lipoprotein to prevent it from being recognized by the receptor (19–23). This, however, does not necessarily eliminate nonspecific protein-mediated uptake (24). To ensure abolishment of protein effects, donors that lack proteins should be used.

Abbreviations: egg PC, egg yolk phosphatidylcholine; LDL, low density lipoprotein; HDL, high density lipoprotein; VLDL, very low density lipoprotein.

The protein-lacking donors most extensively used in studies of surface transfer of cholesterol to cells are vesicles consisting of phospholipids and cholesterol. Vesicles are, however, essentially membrane models and therefore are less useful as lipoprotein models. Lipoproteins are composed of a surface monolayer of polar constituents (phospholipids, cholesterol and apoproteins) surrounding a hydrophobic core of nonpolar lipids, predominately cholesteryl esters and triglycerides (25). Thus microemulsions, i.e., particles with a central core of nonpolar lipids stabilized by a surface monolayer of polar lipids, appear to be a justifiable choice when looking for good protein-free lipoprotein models (26,27). In addition to the results obtainable with vesicles, microemulsions enable us also to examine how the transfer and metabolism of lipoprotein lipids are affected by the composition of the hydrophobic core and by the particle size (different ratios of core and surface lipids).

In this report, the preparation and characterization of well-defined and homogenous-sized microemulsion particles containing cholesteryl oleate as core lipid and cholesterol and egg yolk phosphatidylcholine (egg PC) as surface lipids are described. A size series of these microemulsions was prepared, and the relationship between the size of the microemulsion particles and the rate of cholesterol transfer to cultured cells was investigated.

MATERIALS AND METHODS

Cell culture. Human lung fibroblasts were used. The cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% (v/v) newborn calf serum, 0.1 mM nonessential amino acid solution, 2 mM L-glutamine, 20 mM HEPES, 0.08% (w/v) sodium bicarbonate and 10 μg/ml of gentamicin in a humidifed atmosphere of 95% air and 5% CO_2. The fibroblasts were nontransformed and diploid and were used for experiments between the 8th and 15th passages.

Incubation medium. When it was observed that the microemulsions were unstable in ordinary cell culture media, possibly as a result of their high salt concentrations, a special incubation medium was composed. This medium contained only 1/10 of the amount of NaCl and 1/5 of the amount of KCl originally proposed by Eagle in basal media for cultivation of mammalian cells (28). The concentration of other salts, amino acids and vitamins was in accordance with Eagle's minimum essential medium (29), with the exception that the amount of sodium bicarbonate was 0.80 g/l and that this medium was supplemented with 15 mM HEPES, 10 μg/ml gentamicin and glucose to produce an osmolarity of 300 mOsmol/kg. The osmolarity was determined with a Osmomat 030 osmometer (Gonotec GmbH, Berlin, FRG).

Incubation procedures. For the experiments, cells were seeded in 50-mm diameter Petri dishes and grown to near confluency (200–300 μg cell protein/dish) in 5–7 days. On the day of the experiment, the growth medium was removed, the cell layer was washed once with 3 ml phosphate-buffered saline, and 2 ml incubation medium was added. After a 30-min preincubation at the incubation

temperatures (37 C or 4 C), the labeled microemulsion or LDL preparation was added. At the end of the incubation, the medium was collected and the cell layer was washed twice with cold phosphate-buffered saline containing 0.2% albumin and three times with phosphate-buffered saline without albumin. The cells were then detached by gentle scraping and sedimented by a low-speed centrifugation. The cell pellet was suspended and disrupted in 1 ml distilled H_2O by a short sonication. Aliquots of this suspension were taken for determinations of protein and radioactivity.

Preparation of microemulsions and [^{3}H]labeled LDL. The lipids used for preparation of microemulsions were egg PC, cholesterol and cholesteryl oleate. Stock solutions containing 10 mg/ml of these lipids were prepared in benzene and stored at −20 C. Trace amounts of [^{3}H]cholesterol and [^{14}C]cholesteryl oleate or 1-palmitoyl-2-[^{14}C]oleoyl-phosphatidylcholine were added to these solutions shortly before starting the preparation of microemulsion. Specific activities used were usually about 10,000 CPM/μg for cholesterol, 1,500 CPM/μg for cholesteryl oleate and 200 CPM/μg for egg lecithin.

When calculating the relative amounts of lipids needed to give microemulsion particles of specific sizes, it was assumed that the microemulsions were spherical droplets composed of two distinct phases: a hydrophobic core consisting mainly of cholesteryl oleate stabilized by a surface monolayer 2.0 nm thick (30) composed of PC and cholesterol. The somewhat different densities of the lipids — 1.016 g/ml for PC (31), 1.045 g/ml for cholesterol (32) and 0.96 g/ml for cholesteryl oleate (33) (at 23 C and 1 atm of pressure)—were also considered in these calculations. Since a constant amount of cholesterol (1 mg) and a constant molar ratio of egg lecithin and cholesterol (1:1) were used in the different preparations, it was essential to vary the amount of cholesteryl oleate according to the desired size of the microemulsion particles as indicated in Table 1.

Aliquots of the lipid stock solutions producing the appropriate amount of lipids were pipetted into glass vials. The solvent was evaporated under a stream of nitrogen, and the vials were placed in a vacuum desiccator overnight.

The lipid mixtures were resuspended in 400 μl ethanol/diethyl ether (2:1, v/v). Three hundred μl of this solution was slowly injected into 4 ml, 10 mM Tris/HCl buffer, pH 9.0, through the bottom of a sonication vessel thermostated at 55 C under simultaneous ultrasonic irradiation from an MSE sonifier equipped with a titanium probe (100 watts output). The apparatus used for the sonication is described in greater detail by Lundberg and Suominen (34). The sonication time was varied as follows depending on the type of microemulsion under preparation: 200 nm, 2 min; 150 nm, 5 min; 100 nm, 10 min; 50 nm, 20 min; 20 nm, 35 min.

The crude mixture of microemulsion particles thus obtained was separated according to size in two steps. The first step was a density gradient centrifugation using linear sucrose gradients in Tris buffer. The density of the microemulsion solution was raised using sucrose; 350-μl samples were layered under the gradients (3.5 ml), and centrifugation was performed in a Beckman SW 60 Ti rotor. The centrifugation force, duration and density gradient range differed for the different types of microemulsion, ranging from 10,000 g, 5 min and 0–10% (w/v) sucrose for 200-nm microemulsion particles to 95,000 g, 30 min and 0–30% when preparing 20-nm particles. At the end of the centrifugation, the bottoms of the tubes were punctured and 8-drop fractions were collected. The fractions containing lipids in the appropriate ratios were pooled for the second step of separation, gel filtration.

Ascending gel filtration chromatography was performed on a Sephacryl S-1000 Superfine column (1.6 × 32 cm) at 25 C and with a flow rate of 30 ml/hr using Tris buffer, pH 9.0, as eluent. The samples were applied in volumes of ~ 2 ml, and fractions of 1.45 ml were collected. The void volume V_o was determined using killed *Serratia marcescens* bacteria, and K_{av} was calculated from the equation $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the volume of elution and V_t is the total bed volume.

LDL was isolated from fresh pooled human serum by preparative density gradient ultracentrifugation and labeled with [^{3}H]cholesterol using the exchange procedure described previously (18).

Electron microscopy. Microemulsion particles were negatively stained with 2% potassium phosphotungstate, pH 7.4, containing 200 μg/ml bacitracin on Formvar-coated copper grids. The grids were air-dried and examined in a Zeiss EM 109 electron microscope, calibrated with a grating replica.

Solubility. Twenty or 40 mg [^{3}H]cholesterol was added to 200 mg [^{14}C]cholesteryl oleate. This mixture was heated to 55 C and suspended in 3 ml of 10 mM Tris/HCl buffer, pH 9, in 4-ml ultracentrifugation tubes. The tubes were sealed under N_2 gas and incubated for four days under continuous shaking and repeated heating and cooling between 37 C and 55 C. The cholesterol-saturated cholesteryl oleate oil phase was flotated by ultracentrifugation (37 C, 100,000 g, 60 min) in a Beckman SW rotor, and the solubility of cholesterol was determined by radiochemical assay of the floating oil.

Analytical procedures. Cell and LDL protein were determined by the Lowry method as modified by Markwell et al. (35) and lipid phosphorus according to the method of Bartlett (36). Radioactivity was determined with a liquid scintillation counter (1210 Ultrobeta, LKB-Wallac, Turku, Finland) using Lumagel as scintillation fluid. To study the possible exchange of free cholesterol between the microemulsion core and surface phases, a modification of the cholesterol oxidase–phospholipase C method described by Moore et al. (37) was used. Briefly, microemulsion samples containing 10–20 μg free cholesterol were diluted to 1 ml with distilled H_2O, and 1 ml 50 mM potassium phosphate buffer, pH 7.7, containing 0.2 U phospholipase C and 1.3 mM $CaCl_2$ was added. This mixture was incubated at 37 C for 90 min. Then, 0.2 U cholesterol oxidase in 1 ml phosphate buffer was added, and the incubation was continued for 30 min. The incubation was stopped by adding 2 ml of cold methanol and the produced cholest-4-en-3-one was assayed spectrophotometrically at 240 nm against the reagent blank. The standard curve was constructed using 0–50 μg cholestenone in reagent solution. Total free cholesterol was assayed in the same way with the exception that 1% sodium taurocholate was added to the phosphate buffer to disrupt the microemulsion and solubilize free cholesterol. To determine the possible vesicle contamination of the microemulsion preparation, the 6-carboxyfluorescein method

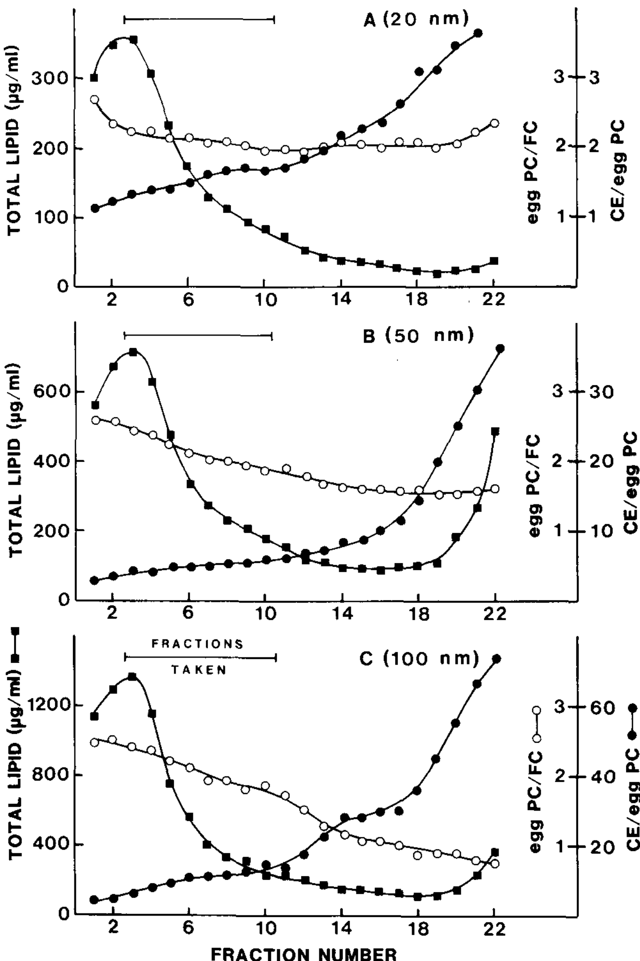

FIG. 1. Density gradient centrifugation of egg PC–cholesterol–cholesteryl oleate microemulsions. Preparation of particles: (A) 20 nm, (B) 50 nm and (C) 100 nm in diameter. Centrifugation was performed in a Beckman SW 60 Ti rotor. Sample volume was 0.35 ml and gradient volume 3.5 ml. Centrifugation conditions were (A) 30 min, 95,000 g, 0–30% (w/v) sucrose; (B) 15 min, 65,000 g, 0–20% (w/v) sucrose; (C) 5 min, 25,000 g, 0–15% (w/v) sucrose gradient. Fractions of 180 µl were collected; fractions taken for further experiments are indicated by horizontal bars. ■, Total amount of lipid in the fractions; ○, egg PC/free cholesterol (FC) weight ratio; ●, cholesteryl oleate (CE)/egg PC weight ratio.

of Weinstein et al. (38) as modified by Lundberg and Suominen (34) was utilized.

Materials. L-3-phosphatidylcholine, 1-palmitoyl-2-[1-^{14}C]oleoyl (57 mCi/mmol) and [7(n)-^{3}H]cholesterol (9.6 Ci/mmol) were purchased from Amersham, (Buckinghamshire, UK). [4-^{14}C]Cholesteryl oleate (59.4 mCi/mmol) was from New England Nuclear (Boston, MA). The radiochemical purities of cholesterol and cholesteryl oleate were found to be >98.5% by thin layer chromatography. Cholesterol and cholesteryl oleate were obtained from Merck (Darmstadt, FRG) and used after recrystallization from 1,2-dichlorethane (cholesterol) or acetone (cholesteryl oleate). Egg L-α phosphatidylcholine, phospholipase C (from *Clostridium perfringens*), and cholesterol oxidase (from *Pseudomonas*) were purchased from Sigma Chemical Co. (St. Louis, MO). Cell growth medium and supplements were obtained from Gibco Europe (Uxbridge, UK) and 4-cholesten-3-on from Fluka AG (Buchs, Switzerland). The Sephadex G-50 and Sephacryl S-1000 Superfine gel media were purchased from Pharmacia Fine Chemicals AB (Uppsala, Sweden), and the *Serratia marcescens* bacteria was a gift from the same firm, Lumagel was obtained from Lumac B.V. (Landgraaf, Holland).

RESULTS

Ultracentrifugation. The distributions of radiolabeled microemulsion particles in the centrifugation tubes after representative centrifugations are shown in Figure 1. Fraction 1 represents the bottom of the tube and fraction 22 the top. As the density of the microemulsion particles was less than unity because of their high content of cholesterol oleate, they had to be layered under the gradient and hence migrated upwards during the centrifugation. The largest (most cholesteryl oleate–rich) particles migrated fastest and the smallest (most dense) migrated slowest. Conditions were chosen to give only a minor migration of the peak of lipid material (see figure). A more effective centrifugation or a steeper gradient rapidly resulted in a creaming of most of the lipid material at the surface of the gradient with risk of coalescence of emulsion droplets. The fractions (indicated in figure) containing lipids in approximately correct ratios (Table 1) to give microemulsions of desired sizes were collected and subjected to gel filtration.

Gel filtration chromatography. Typical gel filtration patterns of preparations aimed at producing microemulsion particles 20 nm, 50 nm and 100 nm in diameter are illustrated in Figure 2. The column elution profile is quite symmetrical for the 20-nm preparation but less so for the 50-nm and 100-nm preparations. The figure also shows that the amount of egg PC relative to that of cholesterol is fairly high both in fractions near V_0 and V_t. This may be a result of the presence of egg PC–rich large multilamellar vesicles eluting near V_0 and small unilamellar vesicles eluting near V_t.

Gel filtration experiments with microemulsions where cholesteryl oleate and cholesterol were radiolabeled showed that the ratio of cholesteryl ester to free cholesterol was very high in fractions near V_0 (data not shown). This indicates the presence of large microemulsions with much core material in these fractions (in addition to the multilamellar liposomes). Regardless, the total amount of lipids in such less desirable lipid complexes was rather small. The major part of the lipids coeluted in the main peak, and the lipid composition throughout this peak elution region was rather homogeneous and in remarkably good agreement with the required (starting) composition (Table 1). Because only a few (2–3) of the peak fractions were collected for incubation experiments, a narrow distribution of microemulsion particle size could be expected. This was confirmed by negative stain electron microscopy showing circular structures (indicating spherical particles) of fairly uniform sizes. Counting 40 particles from a 20-nm preparation gave a size distribution of 23 ± 3 nm (mean ± SD), range 20–27 nm. K_{av} for the fractions taken for incubations (indicated in Fig. 2) were as follows: 20 nm preparation, 0.48; 50 nm, 0.33; 100 nm, 0.19; 150 nm, 0.13; 200 nm, 0.08. The presence of vesicles in a pooled 20-nm preparation was determined by the 6-carboxyfluorescein method and found to be less than 2%.

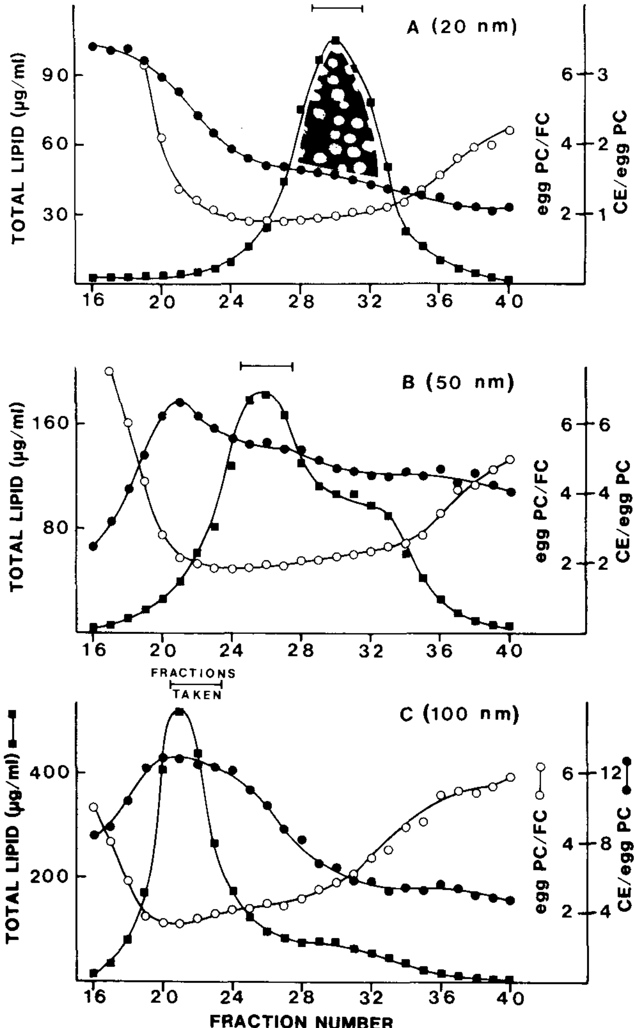

FIG. 2. Gel filtration chromatography of egg PC–cholesterol–cholesteryl oleate microemulsions. Preparation of particles:(A) 20 nm, (B) 50 nm and (C) 100 nm in diameter. After ultracentrifugation and concentration the samples were applied in volumes of ~ 2 ml to a column (1.6 × 32 cm) with Sephacryl S-1000 Superfine gel. The lipid material was eluted with Tris buffer, pH 9.0. Each collected fraction contained 1.45 ml. Fractions pooled for incubations with cells are indicated by horizontal bars. Inserted in (A) is a negative stain electron micrograph of a pooled sample. ■, Total amount of lipid in the fractions; ○, egg PC/free cholesterol (FC) weight ratio; ●, cholesteryl oleate (CE)/egg PC weight ratio.

Cell incubations. When initial incubations of human fibroblasts with microemulsions were performed, an unexpectedly great and rapid decrease of [^{3}H]cholesterol activity in the medium was observed. When this phenomenon was examined further, it appeared to occur also in the absence of cells. The [^{3}H]cholesterol that had disappeared from the medium was found firmly adsorbed to the walls and bottoms of the petri dishes (see figure). The obvious reason for this was a low microemulsion stability in this type of incubation medium (Dulbecco's modified Eagle medium). In an attempt to increase the stability of the microemulsions, an incubation medium with most of the sodium and potassium salts replaced by glucose was composed. In this medium, the microemulsion stability appeared to be satisfactory (Fig. 3B). Whether the increase in microemulsion stability was due to the decrease in salt concentration or the increase in glucose concentration was not further examined. This alteration of the incubation medium composition, however, raised a new question. Do fibroblasts cultured in such a sugar-rich medium deal with lipoproteins and lipoprotein models in the same way as fibroblasts growing under normal conditions? To examine this, an experiment was carried out in which the transfer of [^{3}H]cholesterol from LDL to fibroblasts growing in serum-free cell culture medium of ordinary composition (Dulbecco's MEM) was compared to transfer to fibroblasts cultured in the specially composed sugar-rich incubation medium. For the experiment [^{3}H]cholesterol LDL at a concentration of 25 µg LDL protein/ml medium was added to petri dishes containing 150–200 µg cell protein/dish. The amounts of transferred [^{3}H]cholesterol given as µg/mg cell protein were 3.6 ± 0.2 after 4.5 hr of incubation in Dulbecco's MEM and 3.7 ± 0.3 in the sugar-rich medium. After 9 hr of incubation the amounts were 5.3 ± 0.3 and 5.9 ± 0.5, respectively. (The values are means ± SD from five dish pairs [37 C − 4 C]). Thus the differences in the rates of [^{3}H]cholesterol transfer from the different media were not statistically significant.

Figure 4 compares the transfer of [^{3}H]cholesterol from microemulsion particles of different sizes to cultured human fibroblasts. The transfer rates have been corrected for passive cell surface adsorption by subtraction of transfer rates obtained at 4 C from those at 37 C. The rate of [^{3}H]cholesterol transfer increased with the diameter of the microemulsion particles in the range of 20 to 100 nm. For particles larger than 100 nm in diameter, no further increase in transfer rate was noted. (The curve for 150-nm particles was also determined, but being superimposable with the curves for 100-nm and 200-nm particles, it was not included in Figure 4 for the sake of clarity.)

To elucidate further the mechanism of the transfer process, an experiment with different-sized microemulsions doubly labeled with [^{3}H]cholesterol and [^{14}C]cholesteryl oleate was carried out. The transfer rates of the labeled lipids differed greatly. This shows that [^{3}H]cholesterol and [^{14}C]cholesteryl oleate were transferred separately and independently from the microemulsions to the cultured cells. The results in Table 2 also indicate that the transfer of [^{14}C]cholesteryl oleate is directly dependent on the concentration of this liquid in the incubation medium.

Localization and exchangeability of cholesterol in microemulsion. Because cholesterol is slightly soluble in cholesteryl esters (39–41), a certain proportion of the free cholesterol may be anchored in the hydrophobic core of the microemulsion inaccessible for exchange with cell cholesterol. To test this possibility, the solubility of cholesterol in cholesteryl oleate in the presence of an aqueous phase at 37 C was first determined. The solubility of cholesterol in the oily cholesteryl ester phase was 7.5 ± 0.5% by weight (mean ± SD, n = 4). This implies that a considerable proportion of the microemulsion cholesterol may be localized in the microemulsion core, although solubility in such a microsystem is, due to steric hindrance, not necessarily exactly equal to the solubility in the bulk system examined. Next an effort to establish the proportion of cholesterol that is located in or has access to the surface monolayer of the microemulsions was made. This

TABLE 1

Composition of Lipid Mixture Used for Preparation of Different-sized Microemulsion Particles

Required micro-emulsion particle diameter (nm)	Egg PC (mg)	Cholesterol (mg)	Cholesteryl oleate (mg)	Cholesteryl oleate/egg PC weight ratio	Egg PC/ cholesterol weight ratio
20	1.94	1.0	3.15	1.62	1.94
50	1.94	1.0	10.60	5.46	1.94
100	1.94	1.0	23.00	11.9	1.94
150	1.94	1.0	35.40	18.2	1.94
200	1.94	1.0	48.00	24.7	1.94

TABLE 2

Transfer of [^{3}H] Cholesterol and [^{14}C] Cholesteryl Oleate from Doubly Labeled Microemulsion Particles ca. 20 or 100 nm in diameter to Cultured Human Fibroblasts[a].

Size of ME (nm in ϕ)	Transfer of FC[b] (nmol/mg protein 5 hr.)	Transfer of CO[b] (nmol/mg protein 5 hr.)	CO/FC transfer ratio	CO/FC molar ratio in ME	Ratio of CO transfer (100 nm ME/20 nm ME)	Conc. ratio of CO in medium (= conc. ratio in 100 nm/20 nm ME)
20	1.16 ± 0.12	0.14 ± 0.04	0.12	1.85	$\frac{0.86}{0.12} = 7.2$	$\frac{14.1}{1.85} = 7.6$
100	1.47 ± 0.17	1.25 ± 0.27	0.86	14.1		

ME, microemulsion particle; FC, free cholesterol; CO, cholesteryl oleate.

[a]Cells at a concentration of 200 – 300 μg cell protein/petri dish were incubated in 2 ml incubation medium (see Materials and Methods) containing 5 μg (13 nmol) microemulsion-[^{3}H]cholesterol/ml for 5 hr at 37 C and 4 C.

[b]Calculated from the differences in cell-associated [^{3}H]cholesterol and [^{14}C] cholesteryl oleate radioactivity in cells cultured at 37 C and 4 C. Each transfer value represents the mean ± SD from four dish pairs.

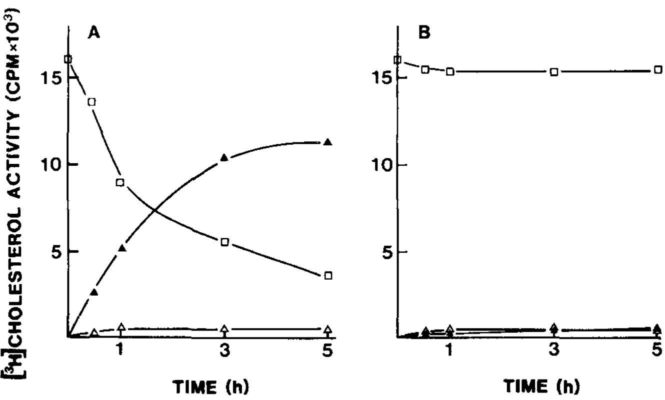

FIG. 3. The effect of incubation medium composition on the stability of microemulsions. Microemulsion particles ca. 100 nm in diameter containing 2 μg [^{3}H]cholesterol were incubated in 2 ml of cell culture medium. In (A) the medium was Dulbecco's modified Eagle medium; in (B) it was the incubation medium generally used in this investigation. This incubation medium was based on Eagle's minimum essential medium, where 9/10 of the NaCl and 4/5 of the KCl were replaced with glucose. Both media were supplemented as described in Materials and Methods. □, [^{3}H]cholesterol radioactivity (CPM) remaining in medium. ▲, [^{3}H]cholesterol activity that could be removed from the dishes by washing twice with 2.5 ml phosphate-buffered saline. ▲, [^{3}H]cholesterol activity that had to be extracted from the walls and bottoms of the dishes with 2.5 ml methanol/chloroform (2:1, v/v). Values are averages from two determinations.

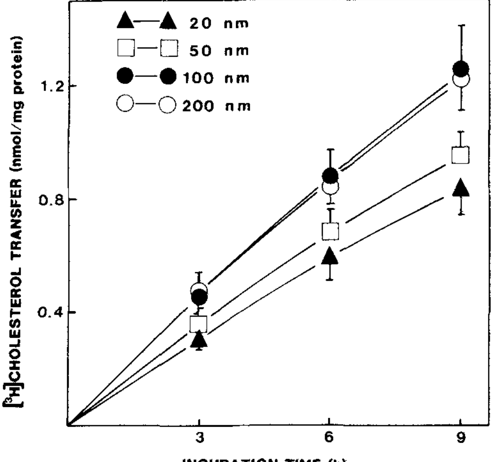

FIG. 4. The effect of microemulsion particle sizes on the transfer of [^{3}H]cholesterol from egg PC–cholesterol–cholesteryl oleate microemulsions to cultured human fibroblasts. Each petri dish contained 200–300 μg cell protein. The cells were incubated at 37 C and 4 C in 2 ml of incubation medium (see Materials and Methods) containing 1 μg (2.6 nmol) microemulsion-[^{3}H]cholesterol/ml. The amount of transferred [^{3}H]cholesterol is calculated from the difference in cell association of radiolabeled cholesterol at 37 C and at 4 C. Error bars represent SD for 6 determinations. Particles were ca. 200 nm (○), 100 nm (●), 50 nm (□), and 20 nm (▲) in diameter.

was done by measuring the susceptibility of cholesterol to oxidation with cholesterol oxidase after the phosphorylcholine head group had been cleaved off with phospholipase C. That phospholipase C action did not cause a disruption of the microemulsion particles was confirmed by the gel filtration elution profile of the microemulsion, which was unchanged after enzymatic treatment.

It was found that the amount of cholesterol oxidized in intact 100-nm microemulsion particles was the same as the amount oxidized in microemulsions disrupted in taurocholate. The values for "surface" cholesterol was 17 $\pm$ 2 μg/0.5 ml (mean $\pm$ SD, n = 5) and for total cholesterol was 18 $\pm$ 3 μg/0.5 ml (mean $\pm$ SD, n = 5). This indicates that the core pool of cholesterol in microemulsions can exchange with the surface pool and is thus available for surface transfer to acceptor structures.

DISCUSSION

These experiment were designed to prepare protein-free lipoprotein models (microemulsions) suited for studies of lipid transfer. For the preparation of microemulsions, a combination of ultrasonic irradiation and injection techniques was used. With such a combined technique two ways of varying the particle or droplet size are available: (i) The size of the particles will vary with the concentration of material in the injected solution. Lower concentrations produce smaller particles. (ii) The size will vary with the time and efficiency of the sonication. Longer and/or more effective sonication produces smaller particles.

The produced microemulsion particles were separated with respect to particle size in two steps: density gradient centrifugation and gel filtration chromatography. The polar surface lipids and hydrophobic core lipids remained associated during these fractionating procedures, which shows that real microemulsions had been produced. That the microemulsion particles in the different-sized preparations were fairly homogenous in size could be verified by electron microscopy. If the partition coefficients, K_{av}, for the fractions selected from the different preparations are plotted against the logarithms of the desired particle volumes, a linear relationship is obtained. This confirms that a size series of the desired type was produced. Chemical and radiochemical assay methods verified that the microemulsion particles had the required lipid compositions. When the results obtained by this combined technique are compared with results obtained utilizing only injection (27) or only sonication (26), it seems as if the combined method gave more homogenous particle sizes than sole injection technique and that the sonication time could be kept shorter than when using sonication technique alone.

The adequacy of these microemulsions as lipoprotein models in studies of physiological transfer processes was tested by incubating them with cultured human fibroblasts. Since different classes of lipoproteins vary considerably in size, an attempt to examine the size effect on transfer rates was made. In the utilization of these types of microemulsion preparations for incubation with cultured cells, a disadvantage that immediately became obvious was their low stability in the cell culture medium. This problem could be solved by decreasing the salt concentration in the medium while at the same time increasing the glucose concentration to maintain iso-osmolarity. This change in medium composition did not have any effect on the uptake of LDL in the cultured cells during an incubation period of 9 hr. It seems, therefore, reasonable that it will not affect the transfer rate of cholesterol either.

The transfer rates obtained in this experiment were 0.09–0.14 nmol cholesterol/mg protein and hour, at a concentration of 1 μg (2.6 nmol) cholesterol/ml incubation medium (Fig. 3). These rates are in good agreement with those previously obtained in this laboratory (23) for receptor-independent cholesterol transfer from HDL and LDL to human lung fibroblasts. The transfer rate of radiolabeled cholesterol from microemulsion preparations to cultured cells increased with the size of the emulsion particles in the range of 20–100 nm diameter. This agrees with results shown previously by Kovanen and Nikkilä (42), who determined relative transfer rates of free cholesterol from different lipoproteins to adipocytes. They found that the rate of exchange decreased with decreasing particle size. Contradictory results have been obtained for surface transfer of cholesterol between vesicles (8,43), showing faster transfer from small vesicles. This indicates that microemulsions may be more appropriate than vesicles as lipoprotein models in studies of lipid transfer. Support for this notion was found in recent studies (Ekman, S., and Lundberg, B., to be published in Biochim. Biophys. Acta) where the maximal [^{3}H]cholesterol transfer rates were about the same from LDL and microemulsions, but vesicles gave transfer approximately twice as fast.

The mechanism for the exchange process has been a matter of much debate. Results supporting both a mechanism involving collisions between the structures in question (1,44,45) and free diffusion of cholesterol through the aqueous phase (6,10,13) have been presented. The results in Table 2 support to some extent the aqueous diffusion mechanism insofar as they show that different components of the microemulsions are transferred to cells at different rates. The transfer rate for the more water-soluble cholesterol was much higher than that of the very unpolar cholesteryl oleate molecule. The results in Table 2 also show that the accumulated [^{3}H]cholesterol activity in the cultured cells was due neither to unspecific adherence of microemulsions to the cell surfaces nor to endocytosis of intact microemulsion particles. In these cases, the ratios of radiolabeled cholesterol and cholesteryl oleate recovered in or with the cells after the incubations would have been approximately the same as the ratios in the incubation medium. Some endocytosis naturally occurs in metabolically active fibroblasts. The fluid phase endocytosis in cultured fibroblasts at 37 C has been determined in this laboratory to ca. 190 nl per mg cell protein and hr (46). A comparison of this value with the transfer data in Table 2 shows that endocytosis may account for only ~ 1% of the cell accumulation of [^{3}H]cholesterol observed in this study.

This paper presents only the influence of microemulsion particle size on cholesterol transfer, but the influence of different core compositions has also been investigated and will be presented elsewhere (Ekman, S., and Lundberg, B., Biochim. Biophys. Acta, in press). Microemulsions of the present type have also been used in studies of factors affecting the lysis of the lipoprotein-like particles in lysosomes (47).

ACKNOWLEDGMENTS

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METHODS

Mass Spectra of the Picolinyl Ester Derivatives of Some Non-Methylene-Interrupted Octadecadienoic Acids

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The electron-impact mass spectra of the picolinyl ester derivatives of 5,12-, 6,12-, 7,12-, 8,12-, 6,10- and 6,11-octadecadienoic acids were obtained. There are features in each that are diagnostic of the position of the terminal double bond, although doubt could exist, with unknown compounds, about the position of the proximal double bond if authentic spectra were not available for comparison purposes. There appear to be features in the spectra of isomers with two and three methylene groups between double bonds that are of value for characterization purposes.
Lipids 22, 664–666 (1987).

One of the more convenient approaches to the location of double bonds in a fatty acid molecule consists in preparing a derivative such as the pyrrolidides (1) or picolinyl esters (2–4), because these give distinctive fragmentations that are generally characteristic of the double bond positions. These and alternative procedures for locating double bonds in unsaturated fatty acids have been reviewed comprehensively elsewhere (5,6).

The picolinyl ester derivatives are especially useful, because they give unambiguous spectra even for polyunsaturated fatty acids. Mass spectra of picolinyl esters have been used to good effect for the identification of unsaturated fatty acids in complex samples of natural origin (7–10). Recently (11) the electron-impact mass spectra of the picolinyl ester derivatives of the complete series of isomeric octadecenoates and methylene-interrupted octadecadienoates were described. Only when double bonds were at the extremities of the molecule were difficulties experienced in deducing the positions of the double bonds. In continuation of these studies, the mass spectra of picolinyl esters of some octadecadienoates with two or more methylene groups between the double bonds are described here. Fatty acids of this type are known to occur in the lipids of marine organisms, especially invertebrates (12,13).

MATERIALS AND METHODS

The all-*cis* 5,12-, 6,12-, 7,12-, 8,12-, 6,10- and 6,11-octadecadienotes were prepared by total synthesis (i.e., from low molecular weight precursors) (14). They were converted to the picolinyl ester derivatives by an improved procedure described elsewhere (9); in brief, the mixed anhydride of each fatty acid with trifluoroacetic acid was reacted with 3-(hydroxymethyl)-pyridine (10-fold molar excess) in the presence of 4-dimethylaminopyridine (1.2 molar proportion) as catalyst. The derivatives were submitted to gas chromatography–mass spectrometry (GC-MS) as described earlier (except that the upper temperature of the column was 10 C lower), i.e., a fused-silica capillary column (25 m × 0.2 mm i.d.), coated with a cross-linked (5% phenyl methyl) silicone (Hewlett

TABLE 1

Relative Abundances (%) of the Molecular Ion and Ions Characteristic of the Picolinyl Moiety and the Positions and Relative Abundances of Ions of Value in Locating Double Bonds in Picolinyl Octadecadienoates (Non-Methylene-Interrupted)

		Ions characteristic of the picolinyl moiety (%)				
Isomer	Molecular ion (%)	m/z = 92	m/z = 108	m/z = 151	m/z = 164	Ions of value in locating double bonds
5,12-	41	100	75	21	26	356 (2%), 342 (4), 328 (18), 314 (15), 300 (6), 274 (2), 260 (8), 246 (4), 232 (16), 218 (5), 204 (2), 178 (2)
6,12-	18	100	51	19	22	356 (1%), 342 (4), 328 (9), 314 (9), 300 (8), 274 (1), 260 (5), 246 (14), 232 (4), 218 (9), 192 (2), 178 (3)
7,12-	21	100	49	12	27	356 (1%), 342 (6), 328 (9), 314 (12), 300 (9), 274 (1), 260 (72), 246 (5), [247 (16)], 232 (1), 206 (7), 192 (4), 178 (4)
8,12-	20	100	41	11	28	356 (1%), 342 (3), 328 (6), 314 (10), 300 (13), 274 (6), 260 (36), 246 (1), 220 (7), 206 (6), 192 (2), 178 (3)
6,10-	15	100	53	19	19	356 (1%), 342 (3), 328 (2), 314 (2), 286 (12), 272 (11), 246 (6), 232 (11), [and 233 (21)], 218 (2), 192 (3), 178 (5)
6,11-	15	100	48	19	21	342 (3%), 328 (3), 314 (4), 300 (11), 286 (5), 260 (1), 246 (42), 232 (3) [and 233 (13)], 218 (3), 192 (3), 178 (4)

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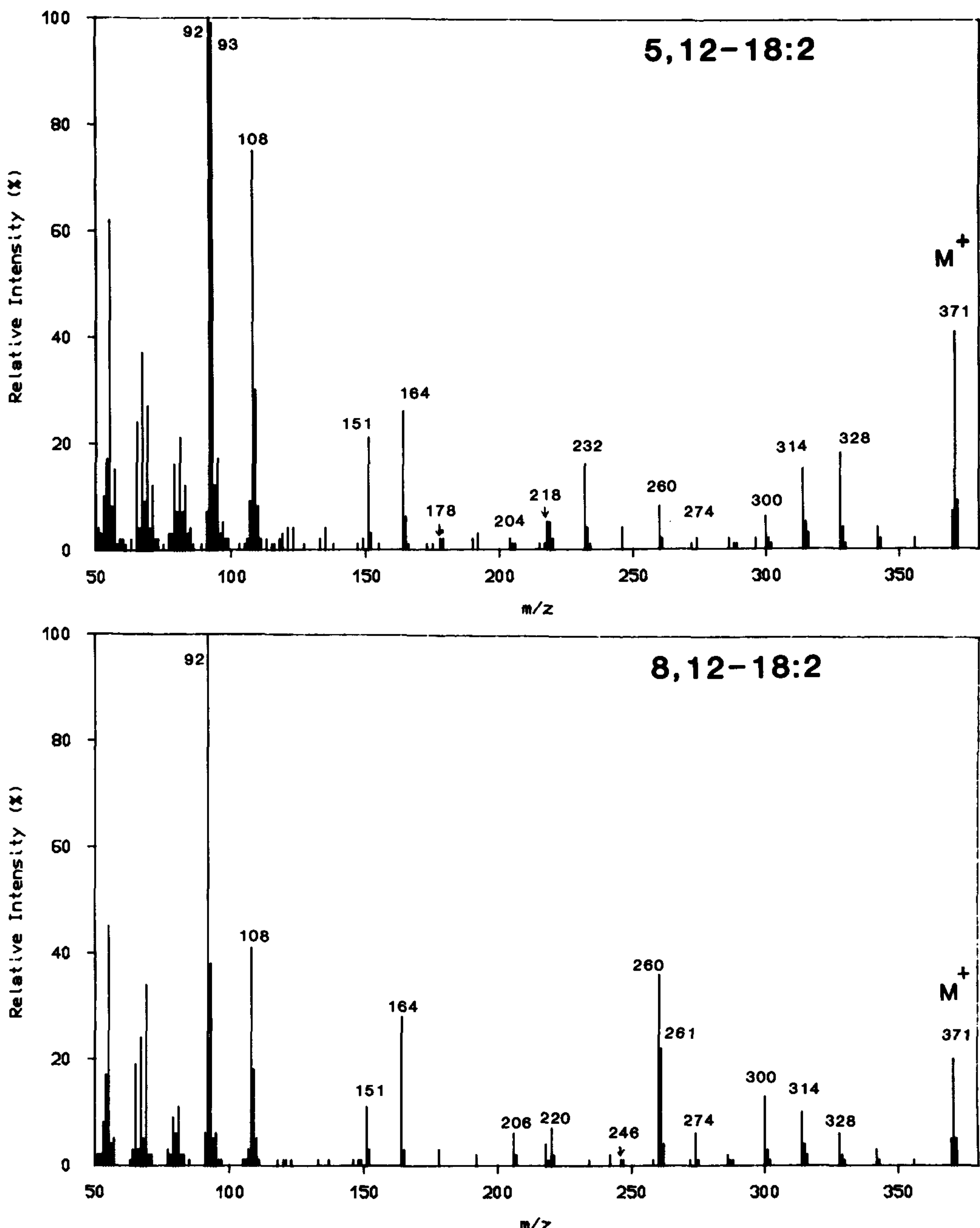

FIG. 1. The mass spectra of picolinyl 5,12-octadecadienoate (top) and picolinyl 8,12-octadecadienoate (bottom).

Packard Ltd., Wokingham, Berks), with helium as carrier gas, was temperature-programmed from 60 C to 220 C at 50 C/min then to 250 C at 1 C/min (7,9,10). The column outlet was connected directly into the source of a Hewlett Packard 5970 Mass Selective Detector, operated at an ionization energy of 70 eV.

RESULTS AND DISCUSSION

Each of the fatty acids was greater than 95% pure as judged from the total ion current trace of the picolinyl esters in the GC-MS experiment, and spectra were taken of material eluting at the centre of each chromatographic peak. The spectra of each of the isomeric picolinyl octadecadienoates are distinctive, and data for some of the ions of diagnostic value are listed in Table 1. The spectra of the picolinyl 5,12- and 8,12-octadecadienoates are illustrated in Figure 1 as examples.

Each isomer gives upon electron impact a prominent molecular ion (at m/z = 371) and abundant ions characteristic of picolinyl esters at m/z = 92 (the base ion in each, as for most picolinyl esters examined to date), 108, 151 and 164.

In the spectrum of the 5,12-isomer (Fig. 1, top), there are significant ions at m/z = 356 (M-CH_3), then with gaps of 14 atomic mass units (amu) separating the ions at m/z = 342, 328, 314 and 300, representing cleavage at successive methylene groups, followed by a gap of 26 amu

for the terminal double bond to m/z = 274. A feature of this kind has been found to be diagnostic for double bond positions in a large number of picolinyl esters (2,4–9). Continuing, there are further ions 14 amu apart at m/z = 260, 246, 232, 218 and 204, followed by another gap of 26 amu to 178 for the proximal double bond. Unfortunately, there are also ions present that could lead, if the structure were not known, to a suggestion that the double bond might be in position 6.

With the spectrum of the 6,12-isomer, the ions from m/z = 356 to 218 are present as in the previous isomer, so that the position of the terminal double bond is easily located. There are again ions characteristic of a double bond in position 6, although *a priori* it is possible to suggest that the proximal double bond is in position 7.

The distinctive feature of the spectrum of the 7,12-isomer (three methylene groups between the double bond) is a particularly abundant ion at m/z = 260 (72%), representing cleavage between carbons 10 and 11 (cf the 6,11-isomer, where there is a prominent ion at m/z = 246). Again the terminal double bond is easily identified, but without access to standards, the position of the proximal double bond might be less easy to determine.

The spectrum of the 8,12-isomer (two methylene groups between the double bonds) also has an abundant ion at m/z = 260, but in this instance as part of a doublet with an ion at m/z = 261 (Fig. 1, bottom). Similarly, the double bond in position 10 of the 6,10-isomer should prove easy to locate from the rules developed earlier, whereas a prominent doublet at m/z = 232/233 appears to confirm that this feature is characteristic of components with two methylene groups between the double bonds.

In summary, the position of the terminal double bond in picolinyl esters of dienes with two or more methylene groups between the double bonds should be easy to identify by employing the principles found to be of value for other unsaturated esters. On the other hand, the position of the proximal double bond could be misidentified as one carbon further from the carboxyl group than is in fact correct. On the other hand, access to spectra of authentic compounds will reduce the chance of error when unknowns are examined by this means. (Several fatty acids of this type have already been identified in the form of the picolinyl ester derivatives in the lipids of invertebrates from the Black Sea [Christie, W. W., Brechany, E. Y., and Stefanov, K., unpublished].) Although it may be misleading to draw general conclusions from only two examples of each, there appear to be features in the spectra of isomers with two and three methylene groups between the double bonds that may serve to remove any dubiety.

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COMMUNICATIONS

Isolation and Quantitation of Lectins From Vegetable Oils

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The factor(s) responsible for the unexplained atherogenicity of peanut oil remain to be elucidated. To this end, we developed a technique to determine if lectin was present in the oil and to quantitate its concentration. This technique was applied to other vegetable oils including corn, soybean, and sunflower. Crude, unprocessed corn and soybean oils were also analyzed for lectin content. The crude oils contained from 858 to 2983 μg lectin per kg, while the refined oils contained 24 to 55 μg/kg of biologically active lectin. The identities of the isolated lectins were confirmed by electrophoresis on SDS-polyacrylamide gels. The biological significance of the presence of lectin in these oils remains to be determined.
Lipids 22, 667–668 (1987).

Peanut oil is more atherogenic than other vegetable oils when fed to laboratory animals as part of a cholesterol-rich or a cholesterol-free diet (1–3). In addition, the atherosclerotic lesions induced are fibromuscular rather than fatty, foam-cell lesions. Studies of alterations in lipid metabolism in animals fed peanut oil have revealed few, if any, significant differences that might account for the increased atherosclerosis observed in animals (4).

Lectins are proteins isolated in significant quantity from crops that provide edible oils. The proteins have potent biological effects mediated via high-affinity binding to specific carbohydrate residues. The presence of lectins in a variety of foods, such as vegetables, fruits, nuts and cereals, has been determined (5) but, to our knowledge, there are no reports of edible oils surveyed for lectin content.

The present investigation was undertaken to develop a technique to extract lectins from vegetable oils, to determine percentage recovery, to verify the identity of the lectins and to quantitate the isolated lectins. We have found lectins present in detectable amounts in processed oils, but these levels are only a few percent of the content found in unprocessed oils.

MATERIALS AND METHODS

The oils used were crude corn, crude soybean, two commercially available brands of processed soybean oil, one processed corn oil, one processed sunflowerseed oil, two processed peanut oils, two peanut oils from special cultivars and randomized (autointeresterified) peanut oil. The density of the oils was determined, and 500-g batches were mixed with 300 ml of petroleum ether in a separatory funnel. One hundred ml of 0.15 M phosphate-buffered saline (PBS) (pH 7.4) was added and mixed well. The phases were allowed to separate, and the aqueous phase was removed and refrigerated. This was repeated four times, and the PBS was pooled. The PBS extracts were concentrated at 4 C using an Amicon ultrafiltration chamber containing a 10,000 MW filter. Protein in the concentrated extracts was quantitated using the Bradford dye binding reagent (Bio-Rad Laboratories, Richmond, CA) (6).

Biological activity of the lectins was determined following the method of Lotan et al. (7), in which human erythrocytes were agglutinated by various concentrations of the PBS extracts. Samples were mixed in cuvettes and left undisturbed for 2.5 hr, and the optical density was measured at 620 nm. Peanut and soybean lectins, used as standards, were purchased from Sigma Chemical Co. (St. Louis, MO). For detection of peanut lectin, the erythrocytes were treated with neuraminidase to remove sialic acid residues (7).

The presence of lectins in oils was verified using 10% acrylamide gels containing sodium lauryl sulfate (8). Samples were electrophoresed under both reducing and nonreducing conditions at 6 mA/gel for 4 hr. Gels were fixed and stained with Coomasie Blue R-250.

Recovery efficiency was measured by dissolving peanut or soybean lectins in their respective oils at 1 mg/100 g by sonication in an ultrasonic bath. The oils were then subjected to the extraction procedure described above, and the biological activity was quantitated at multiple dilutions.

RESULTS

All of the oils tested contained detectable amounts of lectin-like activity. The dye-binding protein assay gave relatively high concentrations compared to the bioassay, suggesting the presence of other proteins as well as lectins (Table 1). One of the samples of processed soybean

TABLE 1

Quantitation of Total Protein and Lectin-like Activity in Crude and Processed Vegetable Oils (μg/kg)

Oil	Total protein	Lectin-like activity
Crude corn	9379	2983
Crude soybean	1926	858
Corn	190	24
Soybean	720	45
Sunflowerseed	849	44
Peanut[a]	580	55
Randomized peanut	204	35
Peanut[b]	120	30
Peanut[c]	154	26
Peanut[d]	206	28

[a]Grown in United States.
[b]Jenkins Jumbo cultivar.
[c]India White cultivar.
[d]Grown in Senegal.

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Abbreviations: PBS, phosphate-buffered saline.

oil contained Tween 80 and gave a very strong color for protein. It was determined that Tween 80 at concentrations as low as 0.0001% reacts in this assay. The other oils were without additives. Almost one-third of the protein in crude corn oil had lectin-like activity, but this was reduced by greater than 99% in the refined product. Analysis of the refined oils by colorimetric protein assay yielded values in the 200–850 μg/kg range, while the bioassay results for lectin gave results in the range of 24 to 45 μg/kg.

Recovery efficiency was determined to be 10%, with a range of replicate experiments from 9.4% to 10.1%. This figure represents recovery of biological activity, not merely extraction efficiency. Factors that may interfere with recovery of biological activity include addition of the solvent to the oils, which facilitated phase separation, or instability of the lectins during the extraction and concentration procedures.

Gel electrophoresis of the PBS extracts revealed bands in the samples from peanut, soybean and corn oils, which corresponded to published molecular weights of lectins from these plants (9–11). It is unclear why no protein bands were found upon electrophoresis of sunflowerseed oil, since the protein recovred by extraction was similar to that from the other oils.

DISCUSSION

The presence of lectins in vegetable oils was previously unsuspected and may have important biological consequences, since lectins are extremely potent compounds. For example, ricin D lectin derived from the castor bean is lethal in mice at 0.001 μg of nitrogen per g body weight; this is several hundred times more toxic than hydrogen cyanide (12). Therefore, the finding of microgram quantities of lectins in these oils may not be inconsequential in the intestine and other organs if they are absorbed from the gut. The presence of soy protein, but not lectin, in lecithin, margarine and oil has been reported (13).

Lectins from peanut, wheat germ and jack bean bind to rabbit aortic smooth muscle cells in vitro (14); several lectins bind to the endothelium of rat cerebral arterioles, but prior neuraminidase treatment is necessary to expose peanut lectin binding sites and accentuates soybean lectin binding sites (15). The lack of neuraminidase treatment may explain why peanut lectin does not consistently stain cellular structures in atherosclerotic arteries (16).

It is important to realize that lectins have specific affinities for sugar residues. Peanut lectin has specificity for D-galactose residues and preferentially for the α-D-Gal(1-03)-D-Gal NAc sequence, which is shared only by the lectin from the mushroom *Agaricus bisporus.* This sequence is expressed in GM_1 ganglioside found in myoblasts from rats and chicks (17,18). The presence of GM_1 on arterial smooth muscle cells may be a reason that peanut oil induces fibromuscular arterial lesions when fed in a hypercholesterolemic diet in contrast to other polyunsaturated fats that induce fatty lesions. This leads to the hypothesis that small amounts of peanut lectin, in the presence of hypercholesterolemia, could enhance proliferation of arterial smooth muscle cells. Lectins also bind to various cellular components of the immune system. Since macrophages are a major cell type in fatty streaks of experimental animals and humans (19,20), and these cells are scant in lesions induced by feeding peanut oil, the possibility exists that peanut lectin inhibits macrophage recruitment to the arterial wall during atherogenesis.

Among the peanut oils studied, the most atherogenic variety (1, unpublished results) contains the most lectin, and when it is randomized the lectin concentration is reduced by half. Randomization of peanut oil reduces its atherogenicity to that of corn oil (2). This suggests that the lectin content of peanut oil correlates with its atherogenic potential.

Although it is unlikely that lectins in the quantities found in these edible oils are of detriment in the human diet, it is possible that when the oils are fed as the sole fat source to experimental animals, the lectin content plays a role in some pathophysiologic effects. Further work is in progress in our laboratory to study the role of lectins in atherosclerosis.

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Deconjugation of Bile Acids by Human Intestinal Bacteria Implanted in Germ-Free Rats

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Fecal bile acids in germ-free rats were analyzed after inoculation with *Bacteroides vulgatus, Bifidobacterium longum, Escherichia coli* or *Clostridium ramosum*. *B. vulgatus* preferentially deconjugated tauro-β-muricholic acid and *B. longum* taurocholic acid. *C. ramosum* deconjugated both bile acids, but *E. coli* deconjugated neither. 7α-Dehydroxylation of bile acids was negligible even after 18 days of inoculation, but a small amount of 7-oxo-bile acids, less than 5%, was formed. Fecal excretion of bile acids increased after inoculation with *B. vulgatus, B. longum* and *C. ramosum*, but not with *E. coli*.
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Biotransformation of bile acids by intestinal bacteria includes deconjugation, desulfation, dehydroxylation, oxidation-reduction and epimerization (1-3). Of these, deconjugation activity is the most commonly observed (4), but it is not yet known whether the reaction is common to different bile acids. The present paper reports that deconjugation activity differs not only with the strain of bacteria but also with the structure of the bile acid.

MATERIALS AND METHODS

Germ-free Wistar male rats (20-37 wk old) bred in our laboratory were housed under germ-free conditions and inoculated orally with four organisms, *Bacteroides vulgatus, Bifidobacterium longum, Escherichia coli* and *Clostridium ramosum,* originally isolated from normal human feces, in which these organisms represented the predominant strain of each group. Each organism was cultured for 72 hr at 37 C in GAM semifluid media (Nissui Co., Tokyo, Japan); after thorough mixing, a 1-ml aliquot was administered to each rat.

All rats were fed a commercially available chow diet (Oriental CMF diet, Oriental Kobo Co., Tokyo, Japan) sterilized by radiation. Feces from each rat were collected at 2-day intervals, and a portion was utilized to determine bacterial concentrations (5). The remaining feces were dired to a constant weight and utilized for bile acid analysis.

Feces were ground, and a portion (usually 1 g) was extracted three times with 20 ml of absolute ethanol for 1 hr at 90 C (6,7). The extract was filtered and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 10 ml of 90% ethanol, and a portion of this solution (usually 1 ml) was subjected to PHP-LH-20 column chromatography to separate bile acids into their free and three conjugate forms—glycine conjugates, taurine conjugates and sulfates (8). The conjugate fractions were hydrolyzed by solvolysis and/or alkaline hydrolysis, and the bile acids in the hydrolysates were extracted with diethylether after acidification with hydrochloric acid solution. The bile acids were methylated with freshly prepared diazomethane and then trifluoroacethylated with trifluoroacetic anhydride. The bile acid derivatives were quantified by gas liquid chromatography utilizing a Shimadzu gas chromatograph Model GC-7A (Shimadzu Co., Kyoto, Japan) equipped with a flame ionization detector. A glass column (1.6 m × 3 mm i.d.) packed with 1.5% QF-1 or 1.5% AN-600 on 80-100 mesh Gas Chrom Q was used. The operation temperatures were 235 C and 210 C for the 1.5% QF-1 and 1.5% AN-600 columns, respectively, and 290 C for the detector (6,7).

RESULTS

Table 1 shows fecal bacterial concentrations in gnotobiote rats examined on days 7 and 14 after inoculation. The number was around 10^{10} in all groups. The total amounts of fecal bile acids excreted in a day and the percentages of free forms and conjugates are given in Table 2. All the bile acids in germ-free rats were conjugated with taurine, and neither glycine conjugates nor sulfates were detected. *B. vulgatus, B. longum* and *C. ramosum* formed free bile acids to some extent, but *E. coli* did not.

The fecal bile acid composition is given in Table 3. Major bile acids in germ-free rats were cholic and β-muricholic acids; they comprised about 90% of the total bile acids. Chenodeoxycholic acid was present at about 5%. In gnotobiote rats, the values for cholic and β-muricholic acids were not changed markedly, but some oxo-bile acids, such as 3α,12α-dihydroxy-7-oxo- and 3α-hydroxy-7-oxo-5β-cholanoic acids, were detected in amounts of less than 6-7%. The total amounts of bile acids in feces increased in rats inoculated with *B. vulgatus, B. longum* and *C. ramosum,* but not in those with *E. coli.*

Table 4 shows the percentages of the free forms of each cholic acid and β-muricholic acid in the feces collected before (day 0) and after inoculation of bacteria on days 3-4, days 9-10 and days 17-18. *B. vulgatus* formed only 3.8% free cholic acid on days 3-4, but markedly deconjugated tauro-β-muricholic acid, giving a value of 91.7%. These values were almost the same in the other determinations on days 9-10 and days 17-18, though they did

TABLE 1

Fecal Bacterial Concentrations in Gnotobiote Rats

	Bacterial counts (10^N)	
	Day 7	Day 14
B. vulgatus ($10^{8.8}$)	10.5	10.6
B. longum ($10^{8.6}$)	9.5	10.3
E. coli ($10^{9.1}$)	10.0	9.6
C. ramosum ($10^{5.9}$)	9.4	10.3

Numerals in parentheses represent the number of bacteria inoculated.

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TABLE 2

Free and Conjugated Bile Acids in Feces of Gnotobiotic Rats

	Total amounts		Composition (days 9–10)			
	Day 0[a] (mg/day)	Days 9–10[b] (mg/day)	Free form (%)	Taurine (%)	Glycine (%)	Sulfate (%)
B. vulgatus	1.59 ± 0.20[c]	3.51 ± 0.44*	34.66 ± 1.79	65.34 ± 1.79	0	0
B. longum	1.24 ± 0.18	1.98 ± 0.38*	47.88 ± 3.81	52.12 ± 3.81	0	0
E. coli	1.67 ± 0.16	1.49 ± 0.30	0	100	0	0
C. ramosum	1.09 ± 0.16	1.79 ± 0.18*	58.56 ± 3.56	41.44 ± 3.56	0	0

[a]Before inoculation. All bile acids were taurine conjugates.
[b]After inoculation.
[c]Mean ± S.E.M. in 5 rats.
*Statistically significant compared to day 0 ($p < 0.05$).

TABLE 3

Fecal Bile Acid Composition in Germ-Free and Gnotobiotic Rats (Days 9–10)

	Germ-free	*B. vulgatus*	*B. longum*	*E. coli*	*C. ramosum*
Total bile acids (mg/day)	1.59 ± 0.20[a]	3.51 ± 0.44	1.98 ± 0.38	1.49 ± 0.30	1.79 ± 0.18
Cholic acid group (%)					
3α 12α	n.d.	n.d.	2.0 ± 1.50	n.d.	1.3 ± 0.84
3α 7α 12α	63.0 ± 2.76[b]	63.3 ± 3.02	65.5 ± 3.37	61.0 ± 4.88	67.3 ± 3.61
3α 12α 7=O	n.d.	4.8 ± 2.68	2.1 ± 1.46	6.7 ± 2.03	2.8 ± 1.78
Chenodeoxycholic acid group (%)					
3α	n.d.	n.d.	n.d.	n.d.	n.d.
3α 6β 7α	n.d.	1.2 ± 0.24	1.2 ± 0.39	n.d.	1.8 ± 1.08
3α 7α	6.1 ± 0.64	3.8 ± 0.57	4.8 ± 0.83	5.1 ± 0.39	4.7 ± 0.99
3α 6α	n.d.	n.d.	n.d.	n.d.	n.d.
3α 6β 7β	31.0 ± 2.48	22.7 ± 2.18	22.7 ± 5.06	23.6 ± 5.29	22.1 ± 5.29
3α 6α 7β	n.d.	n.d.	n.d.	n.d.	n.d.
3α 7=O	n.d.	3.8 ± 1.56	1.8 ± 0.93	3.4 ± 2.81	n.d.

[a]Mean ± S.E.M. in 5 rats. n.d., Not detectable.
[b]Combined values of free and taurine fractions.

TABLE 4

Percentages of Free Form of Cholic (CA) and β-Muricholic (β-MCA) Acids

	Before inoculation		After inoculation					
			Days 3–4		Days 9–10		Days 17–18	
	CA	β-MCA	CA	β-MCA	CA	β-MCA	CA	β-MCA
B. vulgatus	0	0	3.8 ± 1.0[a]	91.7 ± 2.1	2.8 ± 1.3	80.4 ± 6.4	12.6 ± 0.8	90.8 ± 3.6
B. longum	0	0	79.5 ± 4.0	5.3 ± 0.7	64.2 ± 6.5	5.0 ± 0.6	57.7 ± 4.6	7.1 ± 0.4
E. coli	0	0	0	0	0	0	0	0
C. ramosum	0	0	52.9 ± 5.4	30.4 ± 3.6	57.9 ± 2.6	43.1 ± 4.5	58.8 ± 7.0	54.6 ± 13.8

[a]Mean ± S.E.M. in 5 rats. The values indicate the percentages of the free form of each CA or β-MCA.

vary somewhat. On the contrary, *B. longum* mostly deconjugated taurocholic acid and a little tauro-β-muricholic acid. *C. ramosum* deconjugated both bile acids, but *E. coli* deconjugated neither.

DISCUSSION

Deconjugation activity for conjugated bile acids is found in many species of intestinal bacteria (2,3). Among them, Bacteroides, Streptococcus, Lactobacillus, Eubacterium and some aerobic bacteria deconjugate both taurine and glycine conjugates of bile acids (9–11), but Kobashi et al. (12) demonstrated that *Peptostreptococcus intermedius* deconjugated taurocholic acid, while *Lactobacillus brevis* and *Streptococcus faecalis* II-136 deconjugated glycocholic acid predominantly in vitro. They isolated enzymes that hydrolyzed taurine- or glycine-conjugated bile acids specifically from these bacteria.

The present experiment demonstrated that both *B. vulgatus* and *B. longum* deconjugated taurine-conjugated bile acids, but the former deconjugated tauro-β-muricholic acid in preference to taurocholic acid while the latter deconjugated taurocholic acid preferentially. In addition, *C. ramosum* deconjugated both bile acids, but *E. coli* deconjugated neither. Although it is not known whether these bacteria contain enzymes that hydrolyze tauro-β-muricholic acid or taurocholic acid specifically, these taurine-conjugated bile acids are deconjugated by different intestinal bacteria in vivo, at least in rats.

The total amounts of bile acids in feces increased in rats inoculated with bacteria having the ability to deconjugate bile acids. The fecal bile acid excretion of germ-free rats is lower than (almost half) that of conventional rats (13), due to lack of biotransformation of bile acids by intestinal microflora. Although Kellogg et al. (14) concluded that only deconjugation does not cause an increase in fecal excretion of bile acids, our data suggest that it does increase fecal excretion. We speculate that free bile acids are excreted more rapidly than the conjugates, and adhesion of free bile acids to bacteria or dietary fibers would be a cause for larger excretion of bile acids. We also observed that the cecum was bigger in germ-free rats than in conventional rats, and the size was decreased in rats monoinfected with *B. vulgatus, B. longum* or *C. ramosum* but not with *E. coli.* Although absorption of bile acids from the cecum is considered to be less effective than that from the small intestine, the cecum may play a role in bile acid absorption to some extent in germ-free or *E. coli* gnotobiote rats.

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Phosphocholine Phosphatase and Alkaline Phosphatase Are Different Enzymes in Hamster Heart

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The CDP-choline pathway is the major pathway for the synthesis of phosphatidylcholine in the hamster heart. The formation of phosphocholine from choline was regarded as the first committed reaction in this pathway. We demonstrated earlier that the phosphocholine pool in the heart was substantially less than that found in other tissues, and we observed that a substantial amount of the phosphocholine was hydrolyzed back to choline by a phosphatase. This phosphatase was located in the microsomal fraction of the heart, and unlike alkaline phosphatase, it was not inhibited by amino acids. The pH optima and heat sensitivity of phosphocholine phosphatase were also found to differ from alkaline phosphatase. Phosphocholine did not inhibit the hydrolysis of p-nitrophenylphosphate, but a "mixed type" inhibition of the hydrolysis of phosphocholine was observed in the presence of p-nitrophenylphosphate. Our data support the hypothesis that these two activities originate from separate and distinct enzymes, and we postulate that the cardiac phosphocholine phosphatase may play a role in the regulation of the phosphocholine pool size in the hamster heart.
Lipids 22, 672-676 (1987).

Phosphatidylcholine is the major phospholipid in mammalian tissues, and its biosynthetic pathway via CDP-choline was elucidated by Kennedy (1). In hamster heart the majority of phosphatidylcholine biosynthesis proceeds via the CDP-choline pathway (2). Choline is actively taken up by the hamster heart and is rapidly phosphorylated to phosphocholine by choline kinase (2,3). Phosphocholine is converted to CDP-choline by CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme of the CDP-choline pathway (2,4). CDP-choline is condensed with 1,2-diacylglycerol to form phosphatidylcholine (2,4).

In rat liver, the formation of phosphocholine appears to be the first committed step for the formation of phosphatidylcholine (5). The reaction catalyzed by choline kinase is essentially irreversible, and a relatively large and constant pool of phosphocholine is maintained (5). Moreover, pulse-chase studies in rat liver have clearly demonstrated that CDP-choline is the obligatory product of phosphocholine (6). In the hamster heart, the reaction catalyzed by choline kinase is also irreversible, but the phosphocholine pool is much lower than that found in the liver (2,3). Our earlier work suggests that some of the phosphocholine formed in the heart may be hydrolyzed back to choline by a yet undefined phosphatase (2).

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Due to the relatively small pool size of phosphocholine in the hamster heart, alteration of this pool may play a regulatory role in phosphatidylcholine biosynthesis. Hence, the control of phosphocholine hydrolysis by a phosphatase, in addition to the phosphorylation reaction catalyzed by choline kinase, may be important in the maintenance of the phosphocholine pool in the heart. In this study, we provide strong evidence that the hydrolysis of phosphocholine is catalyzed by a specific phosphatase that is different from the cardiac alkaline phosphatase.

MATERIALS AND METHODS

Phosphocholine, p-nitrophenyl-phosphate and alanine were purchased from Sigma Chemical Co. (St. Louis, MO). [Methyl-^{3}H]choline was obtained from New England Nuclear (Boston, MA). AG1-X8 anion exchange resin was purchased from Bio-Rad. Aqueous counting scintillant was obtained from Amersham (Ontario, Canada). Phospho[methyl-^{3}H]choline was synthesized enzymatically from [methyl-^{3}H]choline by the procedure of Paddon and Vance (7). All other biochemicals were of analytical grade and were purchased from Fisher Scientific Chemical Co. (Winnipeg, Manitoba).

Male Syrian golden hamsters of 100 ± 15 g were used throughout the study. The hamsters were maintained on Purina hamster chow and tap water, ad libitum, in a temperature- and light-controlled room.

Hamster hearts were homogenized in 10% 0.25 M sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4. Subcellular fractions were prepared from the homogenate as described previously (8). In brief, the homogenate was centrifuged at 1,000 × g for 10 min, and the supernatant was centrifuged at 12,500 × g for 15 min. The pellet, designated as mitochondrial fraction, was washed with buffer A, recentrifuged and resuspended in buffer B. The supernatant was centrifuged at 100,000 × g for 60 min. The resultant pellet, designated as microsomal fraction, was washed with buffer A, recentrifuged and resuspended in buffer B. Buffer A contained 0.15 M Tris-HCl, pH 8.0, and buffer B contained 0.25 M sucrose, 10 mM Tris-HCl, pH 7.4. The purities of the mitochondrial and microsomal fractions were analyzed by marker enzymes. The mitochondrial fraction was contaminated with 10% of microsomal material and the microsomal fraction contained 5% mitochondrial particles (8). Protein was determined by the method of Lowry et al. (9).

Alkaline phosphatase activity was determined by a modified procedure of Cox and Griffin (10). The assay mixture contained 0.4 M Tris-HCl, pH 10, 1 mM $MgCl_2$, 10 mM p-nitrophenyl-phosphate and 10 µl of the microsomal fraction in a total volume of 0.5 ml. The reaction mixture was incubated at 37 C for 30 min, and the reaction was terminated by addition of 1 ml 0.25 N NaOH. The amount of p-nitrophenol formed was determined by absorbance at 410 nm, with the aid of p-nitrophenol standards. The reaction mixture without the addition of microsomal fraction was used as control.

Phosphocholine phosphatase activity was determined by the formation of labeled choline from labeled phosphocholine (7). The reaction mixture contained 0.1 M Tris-HCl (pH 9.0), 10 mM $MgCl_2$, 10 mM phospho-[methyl-^3H]choline and 30 μl microsomal fraction in a total volume of 0.1 ml. The reaction mixture was incubated at 37 C for 30 min, and the reaction was terminated by boiling the tubes for 2 min. The reaction mixture without the addition of microsomes was used as control. After the termination of the reaction, 1 ml of water was added to the assay mixture, and the entire mixture was applied to a AG1-X8 anion exchange column (1.0 ml bed volume). Choline in the reaction mixture was washed from the column with 10 ml of water, and the radioactivity in a 2-ml aliquot of the eluant was determined. Radioactivity in the sample was determined by liquid scintillation counting using channels' ratio calibration method.

Results in this study are depicted as mean ± S.D. (number of experiments). A two-tailed Students' t-test was used for determination of significance. The level of significance was defined as $P < 0.05$. Results of kinetic inhibition of phosphocholine phosphatase and alkaline phosphatase are depicted as the mean of two separate experiments. Kinetic data were depicted on the basis of least squares analysis of data points.

RESULTS

The majority of alkaline phosphatase (68%) and phosphocholine phosphatase (72%) activities were found to be located in the microsomal fraction of the hamster heart, with the remainder in the mitochondrial fraction. As previously indicated (11), the pH optimum for microsomal alkaline phosphatase was found to be between 9.75 and 10.25, depending on the substrate concentration. In the presence of 10 mM p-nitrophenyl phosphate, the enzyme depicted a pH optimum of 10.0 (Fig. 1). The pH optimum for phosphocholine phosphatase was 9.0 and remained unchanged at different substrate concentrations (Fig. 1).

To discern the possible difference between these two activities in the hamster heart, the effects of L-phenylalanine and L-alanine on alkaline phosphatase and phosphocholine phosphatase activities were investigated. L-Phenylalanine (10–25 mM) inhibited both activities, but a higher degree of inhibition was obtained with alkaline phosphatase than that of phosphocholine phosphatase (Table 1). L-Alanine at 25 and 50 mM caused significant inhibition of alkaline phosphatase activity, but did not inhibit phosphocholine phosphatase (Table 2). The nature of inhibition of alkaline phosphatase by L-alanine was further investigated. The activities of the enzyme at different substrate levels in the absence and presence of 25 and 50 mM L-alanine were determined, and the results are depicted in a double-reciprocal plot. The kinetic studies revealed that the inhibition by L-alanine was essentially uncompetitive (Fig. 2).

The possibility of the two activities sharing the same catalytic site was studied by a kinetic approach. In the presence of phosphocholine (1–50 mM), alkaline phosphatase activities were not affected (Table 3). However, the presence of p-nitrophenylphosphate (1–50 mM) caused significant inhibition of phosphocholine phosphatase activities (Table 3). In the presence of 0.1 and 0.2 mM p-nitrophenylphosphate, a "mixed-type" of inhibition on phosphocholine phosphatase activity was observed (Fig. 3).

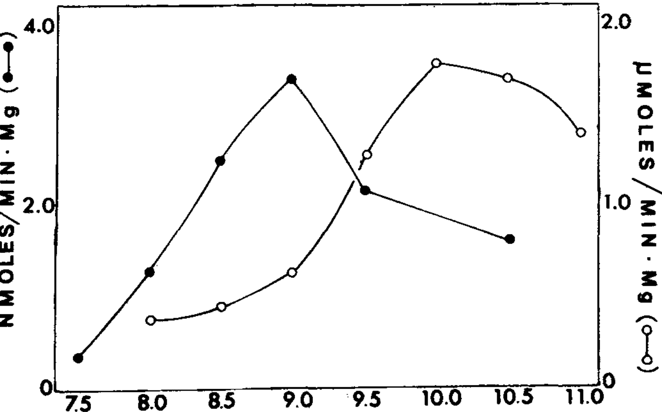

FIG. 1. The pH profiles of phosphocholine phosphatase and alkaline phosphatase. The pH profiles of phosphocholine phosphatase (●) and alkaline phosphatase (○) were determined in the presence of 10 mM concentration of phospho-[Me-^{3}H]-choline and p-nitrophenyl-phosphate, respectively. The points represent the mean values obtained from two separate sets of experiments, each of which was assayed in duplicate.

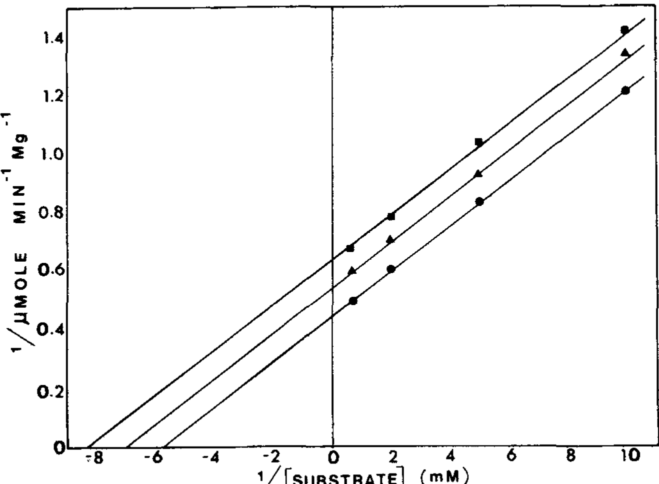

FIG. 2. The inhibition of alkaline phosphatase activity by L-alanine. The hydrolysis of p-nitrophenyl-phosphate in the absence (●) and the presence of 25 mM (▲) and 50 mM (■) L-alanine were determined. The assays were performed as described in Materials and Methods, and 10 μl of microsomal preparation (0.02 mg protein) was used in each assay. The points represent the mean values obtained from two separate sets of experiments, each of which was assayed in duplicate. The lines depicted were obtained from least squares analysis. The correlation coefficient was 0.99 for each line.

The effect of temperature on the activities of alkaline phosphatase and phosphocholine phosphatase in hamster heart microsomes was studied. Microsomal preparations were incubated at 55 C for different time periods, and the enzyme activities were subsequently determined. As shown in Figure 4, treatment at 55 C caused a differential loss of activities between these two enzymes. Incubation of the microsomal preparation for 5 min caused a 30% loss of phosphocholine phosphatase activity, whereas a 10% loss of alkaline phosphatase activity was observed.

TABLE 1

Effect of L-Phenylalanine on Phosphocholine Phosphatase and Alkaline Phosphatase Activities[a]

L-phenylalanine concentrations (mM)	Phosphocholine phosphatase (nmol/min/mg)	Alkaline phosphatase (μmol/min/mg)
0 (control)	3.95 ± 0.06 (3)	2.17 ± 0.21 (3)
1	3.82 ± 0.07 (3)	2.11 ± 0.10 (3)
5	3.34 ± 0.02 (3)[b]	1.71 ± 0.14 (3)[b]
10	3.32 ± 0.09 (3)[b]	1.22 ± 0.08 (3)[b]
25	2.51 ± 0.19 (3)[b]	0.94 ± 0.04 (3)[b]

[a]The hydrolysis of phospho-[Me-^{3}H]choline (10 mM) and p-nitrophenylphosphate (10 mM) by hamster heart microsomal preparation in the presence of L-phenylalanine was determined. The results are depicted as mean ± S.D. (number of experiments).

[b]$p < 0.05$.

TABLE 2

Effect of L-Alanine on Phosphocholine Phosphatase and Alkaline Phosphatase Activities[a]

L-alanine concentrations (mM)	Phosphocholine phosphatase (nmol/min/mg)	Alkaline phosphatase (μmol/min/mg)
0 (control)	3.95 ± 0.06 (3)	2.17 ± 0.21 (3)
1	3.99 ± 0.23 (3)	2.12 ± 0.39 (3)
5	3.97 ± 0.11 (3)	2.16 ± 0.18 (3)
10	3.81 ± 0.35 (3)	2.13 ± 0.10 (3)
25	4.05 ± 0.28 (3)	1.71 ± 0.04 (3)[b]
50	3.92 ± 0.22 (3)	1.65 ± 0.13 (3)[b]

[a]The activities of phosphocholine phosphatase and alkaline phosphatase were determined as described in Table 1 in the presence and absence of L-alanine.

[b]$p < 0.05$.

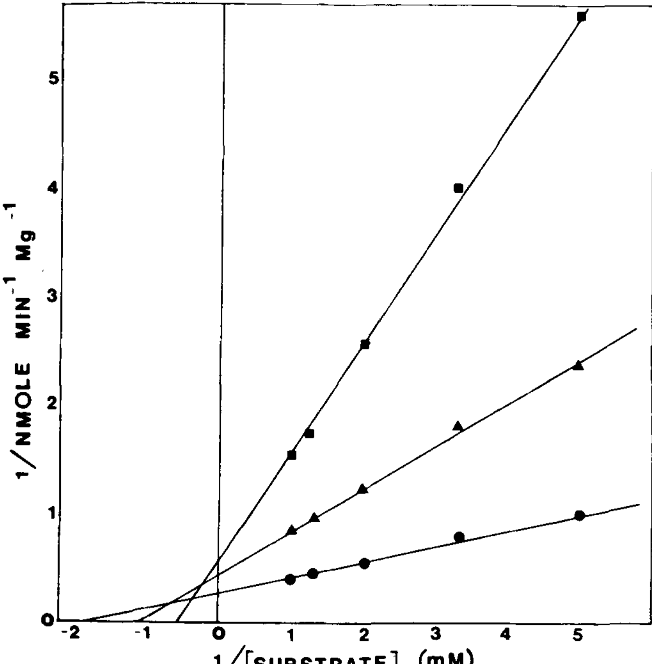

FIG. 3. The inhibition of phosphocholine phosphatase activity by p-nitrophenyl-phosphate. The hydrolysis of phospho-[Me-^{3}H]choline was determined in the absence (●) and presence of 0.1 mM (▲) and 0.2 mM (■) p-nitrophenyl-phosphate. The assays were performed as described in Materials and Methods, and 30 μl of microsomal preparation (0.06 mg protein) was used in each assay. The points represent the mean values obtained from two separate sets of experiments, each of which was assayed in duplicate. The lines depicted were obtained from least squares analysis, and the correlation coefficient was 0.99 for each line.

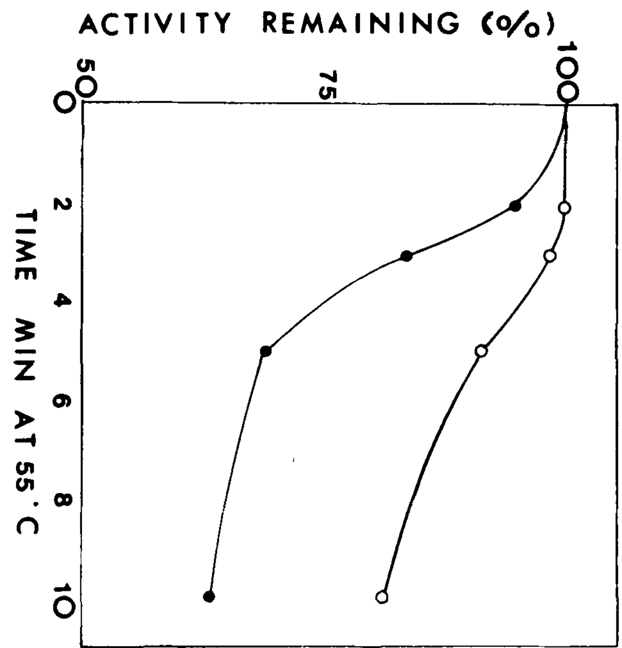

FIG. 4. The effect of incubation at 55 C on phosphocholine phosphatase and alkaline phosphatase activities. Microsomal preparations were incubated at 55 C for 0–10 min. The ability of the incubated microsomal preparation to hydrolyze p-nitrophenyl-phosphate (○) and phospho-[Me-^{3}H]choline (●) was determined. The assay conditions were as described in Figs. 2 and 3. The points represent the mean value of two separate sets of experiments, each of which was assayed in duplicate.

DISCUSSION

The objective of this study was to examine whether phosphocholine phosphatase and alkaline phosphatase are the same enzyme in hamster heart and to determine whether these two activities were subjected to the same metabolic regulation. It is clear from our study that the two activities in the hamster heart microsomes are modulated differently in vitro. The difference in pH profiles and responses to heat treatment and to various inhibitors all suggest that the two activities originate from two separate and distinct enzymes.

Alkaline phosphatase from mammalian sources exists in three distinct categories: the placenta type, the intes-

TABLE 3

Effect of p-Nitrophenylphosphate on Phosphocholine Phosphatase and Phosphocholine on Alkaline Phosphatase Activities[a]

p-Nitrophenylphosphate (mM)	Phosphocholine (mM)	Phosphocholine phosphatase (nmol/min/mg)	Alkaline phosphatase (μmol/min/mg)
0 (control)	0	3.95 ± 0.06 (3)	2.17 ± 0.21 (3)
1	—	1.07 ± 0.26 (3)[b]	
5	—	0.51 ± 0.06 (3)[b]	
10	—	0.52 ± 0.20 (3)[b]	
25	—	0.23 ± 0.09 (3)[b]	
—	1		2.44 ± 0.12 (3)
—	5		2.33 ± 0.31 (3)
—	10		2.41 ± 0.10 (3)
—	25		2.40 ± 0.11 (3)
—	50		2.30 ± 0.04 (3)

[a]The hydrolysis of phospho-[Me-^{3}H]choline in the presence of p-nitrophenylphosphate and the hydrolysis of p-nitrophenylphosphate in the presence of phosphocholine were determined. Assay conditions were the same as described in Materials and Methods.

[b]$p < 0.05$.

tinal type and the liver-bone-kidney type (11). They are different from each other by their responses to uncompetitive inhibition by specific amino acids, heat stability and pH optima at specific substrate concentrations. Previous studies have clearly demonstrated that the cardiac alkaline phosphatase belongs to the liver-bone-kidney type (12,13). It has also been reported that the liver-bone-kidney type in the heart may exist in multiple molecular forms, and these forms may have different properties (11–13). Hence, it can be argued that the observed differences between phosphocholine phosphatase and alkaline phosphatase in this study may result from a broad specificity of one specific form of cardiac alkaline phosphatase. However, this argument is not supported by the kinetic data of phosphocholine phosphatase in the presence of p-nitrophenylphosphate. Further evidence against this supposition was obtained from the inhibition studies of alkaline phosphatase by phosphocholine.

Since the conversion of phosphocholine to CDP-choline is the rate-limiting step in the CDP-choline pathway (2,14), the maintenance of the phosphocholine pool is important to facilitate the rate-limiting role of the CTP:phosphocholine cytidylyltransferase. In the hamster heart, the observed turnover of phosphocholine by dephosphorylation (2) implies that phosphocholine phosphatase may play a significant role in the control of phosphatidylcholine biosynthesis via the regulation of the phosphocholine pool. If phosphocholine phosphatase indeed contributes to the regulation of phosphatidylcholine biosynthesis, its modulation and metabolism should be distinct from the alkaline phosphatase. Our study reveals that, unlike alkaline phosphatase (15), phosphocholine phosphatase in the heart may not be subjected to modulation by alanine and other amino acids (Hatch, G.M. and Choy, P.C., unpublished results). The ability to hydrolyze phosphocholine in vitro had also been reported in the liver (16), intestinal cells (17) and HeLa cells (7), but such reaction was postulated to be of limited importance in the regulation of phosphocholine pool or phosphatidylcholine biosynthesis in these tissues (7). However, the pool of phosphocholine in the heart (0.24 mM) is much lower than that found in the liver (1.3 mM) or HeLa cells (1.8 mM) (14). In the heart, the diminished pool size of phosphocholine implies that the metabolism of this important metabolite of the CDP-choline pathway may be regulated differently. At present, the rationale for a relatively small pool of phosphocholine pool in the heart is not explained. It is also not clear if the small phosphocholine pool in the heart can be attributed to the functional role of the cardiac phosphocholine phosphatase. In view of the fact that the apparent Km (0.56 mM) of the enzyme for phosphocholine (Fig. 3) is much greater than the pool of phosphocholine in the heart, it is possible that one of the major functions of the cardiac phosphocholine phosphatase is to prevent the accumulation of a large phosphocholine pool.

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The Activity and Properties of a Hepatic Acid Cholesteryl Ester Hydrolase Obtained from Rats of Different Age Groups

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The activity of lysosomal acid cholesteryl ester hydrolase (acid CEH, EC 3.1.1.13) in rat liver was determined at 3, 5, 7, 10 and 20 wk following birth. The levels of acid CEH activity showed a marked decrease as rats grew older, whereas those of other lysosomal marker enzymes, such as acid phosphatase, β-glucuronidase and cathepsin B and D, showed only a slight decrease. On the other hand, acid CEH activity was detected in all subcellular fractions obtained from rat liver, but the enzyme activity in these fractions did not show the age-related decrease observed in the lysosomal fraction. The results presented here suggest that the marked alteration of lysosomal acid CEH activity that accompanies aging may be related to its possible involvement in the regulation of cholesterol concentration in rat liver.
Lipids 22, 677–679 (1987).

It is well known that hepatic lysosomal acid cholesteryl ester hydrolase (acid CEH) plays an important role in the metabolism of exogenous cholesteryl esters introduced into cells as lipoproteins (1). The activity of the enzyme is lowered under several conditions, such as fasting (2), Wolman's disease (3), thyroidectomy (4) and Triton WR-1339 treatment (5). It has also been reported that acid CEH activity may be an important factor in the accumulation of cholesteryl esters such as that occurring in atherosclerosis (6).

We have recently shown that acid CEH activity varies according to a diurnal rhythm (2). In addition, we have found that a cytosolic protein in rat liver has an inhibitory effect on lysosomal acid CEH activity (7). However, the mechanisms involved in the regulation of lysosomal acid CEH are as yet unknown.

On the other hand, age-related changes in the activities of HMG-CoA reductase (8) and 7α-hydroxylase (9) and in the absorption (10) and turnover (11) levels during cholesterol metabolism have been reported. In the present experiment, we studied the relationship between aging and changes in lysosomal acid CEH activity in rat liver.

MATERIALS AND METHODS

Chemicals and radiochemicals. Cholesteryl [1-^{14}C]oleate (sp act 58.6 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Phenolphthalein glucuronide, phenylphosphate, hemoglobin and α-N-benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA) were purchased from Sigma Chemical Co. (St. Louis, MO).

Animals. Male Sprague-Dawley rats were purchased from Japan Clea Corp. (Tokyo). Rats were housed under controlled lighting (lights on from 06:00 to 18:00) and were given food and water ad libitum.

Preparation of subcellular fractions. For the preparation of subcellular fractions from rat liver, the method of Brecher et al. (12) was employed. Rats were killed by decapitation, and livers were perfused with ice-cold 1.15% KCl solution at 4 C. The tissues were homogenized in 8 vol of ice-cold 0.25 M sucrose/1 mM EDTA/0.01 M Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 1,000 × g for 10 min, and the resulting supernatant was centrifuged at 3,300 × g for 20 min. The 3,300 × g supernatant solution was then centrifuged at 12,000 × g for 35 min. The resulting pellet was rehomogenized in 0.25 M sucrose solution and recentrifuged at 12,000 × g for 20 min. The original 12,000 × g supernatant solution was recentrifuged at 105,000 × g for 60 min.

Acid cholesteryl ester hydrolase assay. The activity of acid CEH was measured by the method of Brecher et al. (12). Preparation of the substrate was performed as described previously (7).

Assay of lysosomal marker enzymes. Cathepsin D was assayed by the method of Hirado et al. (13), and the amount of reaction products was assayed by the method of Lowry et al. (14). Cathepsin B was assayed by the method of Lenney et al. (15), using BANA as the substrate. β-Glucuronidase was assayed using phenolphthalein glucuronide as the substrate. Phenolphthalein liberated from the substrate was measured by the method of Gianetto et al. (16). Acid phosphatase was measured using phenylphosphate as the substrate essentially as described previously (17). Protein concentration was assayed by the method of Lowry et al. (14).

RESULTS AND DISCUSSION

Changes in the activity of lysosomal acid CEH in rat liver were studied over a period extending from 3 to 20 wk after birth. The acid CEH activity showed a gradual reduction as rats grew older, that is, the activity in lysosomes from 20-wk-old rats was decreased to about one-half of the level in 3-wk-rats (Fig. 1).

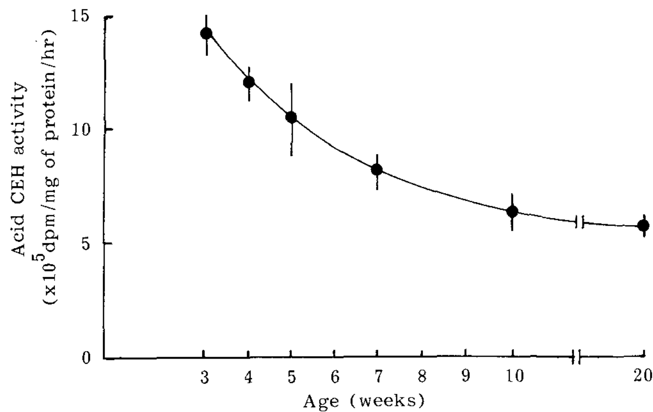

FIG. 1. Acid cholesteryl ester hydrolase (acid CEH) activity during liver development. Standard assay conditions were used as described in Materials and Methods. Values are given as mean ± SEM of six rats.

*To whom correspondence should be addressed.
Abbreviations: CEH, cholesteryl ester hydrolase; BANA, α-N-benzoyl-DL-arginine-2-naphthylamide hydrochloride.

On the other hand, the levels of other lysosomal marker enzymes, such as acid phosphatase, β-glucuronidase and cathepsin B and D, showed little change as rats grew older, and their activities in 20-wk-old rats were about 101%, 86%, 91% and 93%, respectively, of those of 3-wk-old-rats.

It is generally accepted that acid CEH is found in all subcellular fractions from rat liver and that the major proportion of the enzyme activity appears to be associated with lysosomes. We therefore compared the age-related changes occurring in acid CEH activity in various subcellular fractions obtained from both 3- and 20-wk-old rats (Fig. 2). The distribution profile of acid CEH in 3-wk-old rats was similar to that obtained from 20-wk-old rats except for an age-dependent decrease in acid CEH activity observed in the lysosomal fraction. These results suggest that the decrease in acid CEH activity as rats grew older is markedly dependent upon the lysosomal fraction in rat liver and that lysosomal acid CEH may be related to the regulation of cholesteryl ester hydrolysis in liver cells.

Brooks et al. (18) recently reported that the activity of acid triacylglycerol lipase from rat lung was significantly low in the fetus, but increased at birth, and later showed a decline in adulthood. In terms of such an age-related decrease of acid lipolysis in rats, our present results seem to be similar to data on the properties of acid triacylglycerol lipase from rat lung.

It is generally known that the levels of biosynthesis (19) and biodegradation of cholesterol (9,11) are reduced with aging. However, Kritchevsky et al. (20) have reported that the lipolytic activity of rat aorta increases with aging and that the phenomenon may be part of a protective mechanism against the accumulation of cholesteryl esters with aging. In contrast, Brown et al. (1) have reported that the intracellular enzymes located in lysosomes may play a role in regulation of the serum cholesterol level. In our investigations in changes as rats grew older, it is noteworthy that the levels of acid CEH activity were markedly decreased as rats grew older, whereas those of other lysosomal marker enzymes were unaffected by different age groups. This finding suggests that an age-dependent increase in serum and liver cholesterol levels may be partly related to the observed decrease of lysosomal acid CEH activity as rats grew older. In order to confirm this possibility, however, further studies will be required.

On the other hand, several authors have reported that changes in lysosomal acid CEH activity in atherosclerosis could be caused by alterations in the lipid composition and other properties of the substrate vehicle or by substrate dilution at the interface (12,21), that is, enzyme-substrate interaction might be important for the hydrolytic activity of acid CEH. Accordingly, the kinetic parameters of lysosomal acid CEH, given in Figure 3 as Lineweaver-Burk plots, showed apparent Km values of 10 μM and 9.1 μM and Vmax values of 0.33 nmol and 0.16 nmol in 3- and 20-wk-old rats, respectively. Our results thus showed that the affinity of acid CEH, as indicated by the similarity in the apparent Km, did not change as rats grew older. Moreover, protein synthesis is decreased in old animals, as shown by Von Hahn (22) and Mainwaring (23). Furthermore, Yamamoto and Yamamura (10) have reported that decreased protein synthesis in aging animals may cause decreased formation of enzymes involved in lipid synthesis. From these reports, our result suggests that the age-dependent decrease in acid CEH activity may be due to a reduced capacity for de novo enzyme synthesis with aging.

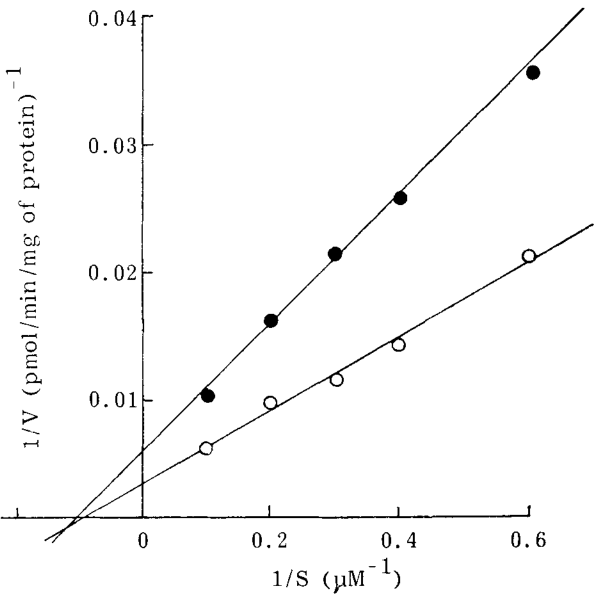

FIG. 3. Lineweaver-Burk plots of acid cholesteryl ester hydrolase. ○, Young rats (3 wk); ●, old rats (20 wk). Standard assay conditions were used as described in Materials and Methods.

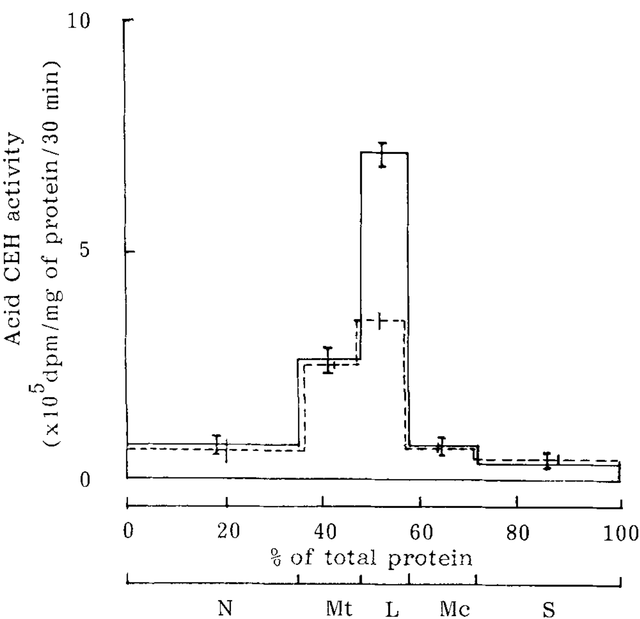

FIG. 2. Subcellular distribution of acid cholesteryl ester hydrolase (acid CEH) activities in livers of 3- or 20-wk-old rats. Results are expressed as specific activities vs cumulative percentage of total recovered protein and are the mean of six experiments. Total recovery of protein was 90%. ————, 3-wk-old rats; - - - -, 20-wk-old rats. Fractions: N, nuclear; Mt, mitochondrial; L, lysosomal; Mc, microsomal; S, supernatant.

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ERRATUM

In the paper "Partial Purification and Characterization of Free and Immobilized Lipases from *Mucor miehei*" by Birgitte Huge-Jensen, Donna Rubano Galluzzo and Robert G. Jensen, Vol. 22, No. 8, pp. 559–565, there were two typographical errors, one giving an incorrect temperature and the second omitting part of a figure legend.

In the Materials and Methods section, the sentence starting on line 10, page 560, should have read:

(b) Step 2: hydrophobic interaction chromatography. A solution (50 ml) containing 2% of the DEAE Sepharose purified powder in 0.2 M ammonium acetate, pH 4.7, was stirred for 30 min at 30 C.

The legend for Fig. 8 should have read:

FIG. 8. Tandem-crossed immunoelectrophoresis, stained with Coomassie Brilliant Blue. Arrows indicate lipases, detected as described in Fig. 2. Applied antibodies as in Fig. 2. (a) Well 1, 5 μg of conA-Sepharose purified lipase A preparation. (b) Well 2, 4.4 μg of phenyl-Sepharose purified lipase B preparation..

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Monohydroperoxides of Linoleic Acid in Endoplasmic Lipids of Rats Exposed to Tetrachloromethane

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An accurate method for the quantitative determination of hydroperoxy and hydroxy fatty acids in liver microsomes is presented which involves the use of ^{18}O-labeled internal standards. The method is employed for the determination of hydroperoxides in rat liver microsomes after aerobic incubation with Fe^{2+}/ADP and in microsomes from animals exposed to 75 mg tetrachloromethane/kg body weight. The amounts found after artificial microsomal "lipid peroxidation" are almost two orders of magnitude larger than those in microsomes from tetrachloromethane-exposed animals.
Lipids 22, 689-697 (1987).

The radical-initiated oxidative degradation of polyunsaturated fatty acids of membrane lipids, commonly referred to as "lipid peroxidation," has been suggested as the underlying mechanism in the toxicity of various xenobiotics such as tetrachloromethane (1), acetaminophen, adriamycin, alcohol, nitrogen oxides, ozone (2), cadmium (3) and 2,3,7,8-tetrachlorodibenzodioxin (4). It has also been discussed as being involved in atherosclerosis (2), hemolytic anemia (5), the pathogenicity of zinc deficiency (6), cancerogenesis and aging (2).

Lipid peroxidation is viewed as the biochemical counterpart to autoxidation of fats. The latter is a process characterized by formation of unpleasant flavors and leads to drying of fats and oils via autocatalytic oxidative degradation and polymerization of polyunsaturated fatty acids (7). More detailed investigations of the reactions and structures involved have recently been initiated (8,9).

The suggestion that lipid peroxidation participates in a pathogenic process is usually based upon the presence of substances in membrane lipids forming with thiobarbituric acid a chromophore with an extinction maximum of 535 nm (10). Other phenomena associated with lipid peroxidation are an increase in the extinction of lipids at 235 nm (11), formation of fluorescent products of undefined structure (12), loss of polyunsaturated fatty acids (13) and expiration of alkanes by animals exposed to lipoperoxidative xenobiotics or conditions (14). Further methods to monitor lipid peroxidation are electron-spin resonance spectroscopy (15) or spectrophotometric detection of chemiluminescence (16). These reflect various side reactions or side products that are usually observed when polyunsaturated fatty acids are subjected to oxidation. Their correlation to pathophysiological derangements is not surprising when lipid membranes are damaged.

Aerobic microsomal incubations usually serve as in vitro models for lipid peroxidation. Such a model can be regarded as a valid analog when similarity in quantitative terms to the effects elicited by an assumed lipoperoxidative xenobiotic in vivo is found. Tetrachloromethane is regarded as a prime example for such a chemical. Indeed, small amounts of alkanes are invariably exhaled by animals exposed to this compound. However, other parameters of lipid peroxidation are often not correlated (17,18).

Formation of fatty acid hydroperoxides is the primary stage in reaction schemes invoked in lipid peroxidation. The purpose of the present work is the identification and quantitative determination of hydroperoxides in microsomal lipids of CCl_4-intoxicated animals. Hydroperoxides decompose rapidly during work-up. Therefore, for quantitative determination they are usually reduced to the corresponding hydroxy derivatives (19,20). This, on the other hand, hampers the individual determination of hydroperoxides and hydroxides. Liquid chromatographic analysis in principle does not require reduction (21,22) but is not sufficiently selective and sensitive to allow the reliable determination of hydroperoxides in complex biological matrices (23-25).

We used gas chromatography and mass spectrometry (GC/MS) with suitable ^{18}O-labeled internal standards. This allows differentiation between hydroperoxy and hydroxy fatty acids and their quantitative determination in vivo. Hydroperoxides in microsomes from a typical in vitro lipid peroxidation experiment and in microsomes from rats exposed to tetrachloromethane have been determined.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from suppliers as indicated: adenosine 5'-diphosphate disodium salt (ADP), isocitrate dehydrogenase 2U/mg and nicotinamide adenine dinucleotide phosphate (NADP) from Boehringer-Mannheim (Mannheim, FRG); 18-oxygen from MSD, (Montreal, Ontario); hydroxy octadecanoic acid from Nu-Chek-Prep (Copenhagen, Denmark); D,L-isocitric acid sodium salt dihydrate, phospholipase A_2 (*Naja naja,* 553 U/mg) and soybean lipoxygenase I (EC 1.13.1.13.) from Sigma Chemical (St. Louis, MO); platinum IV oxide from Ventron (Karlsruhe, FRG); trimethylchlorosilane from Pierce (Rotterdam, The Netherlands); and acetic acid, *bis*-trimethylsilyl-acetamide, calcium chloride dihydrate, citric acid monohydrate, diethylether, ethylenediamine tetraacetic acid, hexane, iron (II) sulfate heptahydrate, isopropanol, linoleic acid, magnesium chloride hexahydrate, methanol, phenobarbital sodium, potassium chloride, potassium hydroxide, pyridine, saccharose, silica gel Si-60, sodium borohydride, anhydrous sodium sulfate, disodium tetraborate decahydrate, toluene, triphenylphosphine and *tris*-hydroxymethyl-amino methane from Merck (Darmstadt, FRG).

In vitro experiments: pretreatment of animals and preparation of microsomes. Male Sprague-Dawley rats weighing 300-400 g were purchased from Ivanovas (Kisslegg, FRG). Before the experiments, they were kept for 1 wk at a constant day-night cycle of 24 hr for acclimatization, with free access to food (Altromin, Lage, FRG) and water.

*To whom correspondence should be addressed.
Abbreviations: GC/MS, gas chromatography/mass spectroscopy; ADP, adenosine 5'-diphosphate disodium salt; NADP, nicotinamide adenine dinucleotide phosphate; PTFE, polytetrafluoroethylene; HPLC, high performance liquid chromatography.

For induction of drug-metabolizing enzymes, the animals received one intraperitoneal injection of 80 mg phenobarbital/kg body weight dissolved in 1 ml distilled water and for the following 5 days 0.1% phenobarbital in the drinking water. After starvation for 24 hr, they were killed by decapitation. The livers were removed, cut into small pieces, rinsed twice with cold saline and homogenized in Tris/KCl buffer (Tris/HCl, pH 7.4, 50 mmol/l; KCl, 120 mmol/l) containing 250 mmol/l saccharose and 1 mmol/l EDTA-sodium salt. Microsomes were prepared by fractionated centrifugation (26). The pellet was suspended in Tris/KCl buffer, and the microsomes were sedimented again and resuspended to a protein concentration of 10 mg/ml. The following experiments were performed immediately afterwards.

Protein determination. Protein was determined according to Peterson's method (27). A calibration curve was constructed with bovine serum albumin. Cytochrome P-450 was determined according to Omura and Sato (28), with aliquots diluted to a concentration of 2 mg microsomal protein/ml.

Aerobic incubation of microsomes with Fe^{2+}/ADP. Microsomal suspension, 0.8 ml, corresponding to 8 mg microsomal protein was incubated at 37 C for 5 min in the presence of 0.4 ml sodium isocitrate solution (80 mmol/l), 0.8 units isocitrate dehydrogenase, 1.6 ml Tris/KCl buffer (pH 7.4, as above) and 0.4 ml magnesium chloride solution (33 mmol/l). After addition of 0.4 ml of a solution of NADP (10 mmol/l in Tris/KCl buffer) and 0.4 ml of a mixture of $FeSO_4$ (0.2 mmol/l) and ADP (20 mmol/l), the microsomes were incubated for another 15 min. The reaction is stopped by adding 1 ml sodium-EDTA (50 mmol/l) and cooling to 0 C. Aliquots of 1 ml are removed for quantitative determination of hydroperoxy- and hydroxy-octadecadienoic acid as described below.

Exposure of rats to tetrachloromethane. The rats received a dose of 75 mg/kg tetrachloromethane by inhalation (29). After 6 hr, the animals were killed and microsomes were prepared.

Preparation of isotope-substituted standards: [$^{18}O_2$]hydroperoxyoctadecadienoic acid. The equipment used is shown in Figure 1. The volume of the reaction vessel was 150 ml. The two standard ground joints were equipped with screw-cap adapters (a,b) with silicone-rubber seals laminated with polytetrafluoroethylene (PTFE). A pointed glass rod of 6 mm diameter (c) and the [$^{18}O_2$] gas ampoule were inserted as shown. The adapter *(e), fused directly to* the vessel, was closed with a PTFE-laminated silicon septum. A dropping funnel (g), 250 ml volume, was connected with polyethylene tubing (4 mm id) to the stop-cock (f) of the incubation vessel. Linoleic acid (0.8 mmol) was suspended by sonication for 5 min in 120 ml borate buffer (125 mmol/l, pH 9), filled into the dropping funnel and purged with a stream of argon for 30 min.

Both stop-cocks (f and h) were opened and the vessel was gently filled, the adapter (b) being lifted to let the air escape. The stop-cock (h) of the dropping funnel was closed, and the funnel was rinsed with 20 ml borate buffer. After replacement of oxygen for argon, the stop-cock was opened again, and the buffer was transferred to the vessel as described before. This was repeated. Any residual air entrapped in adapter (b) was removed by turning the vessel; slight tapping let the air escape via the dropping funnel. The funnel was filled again with

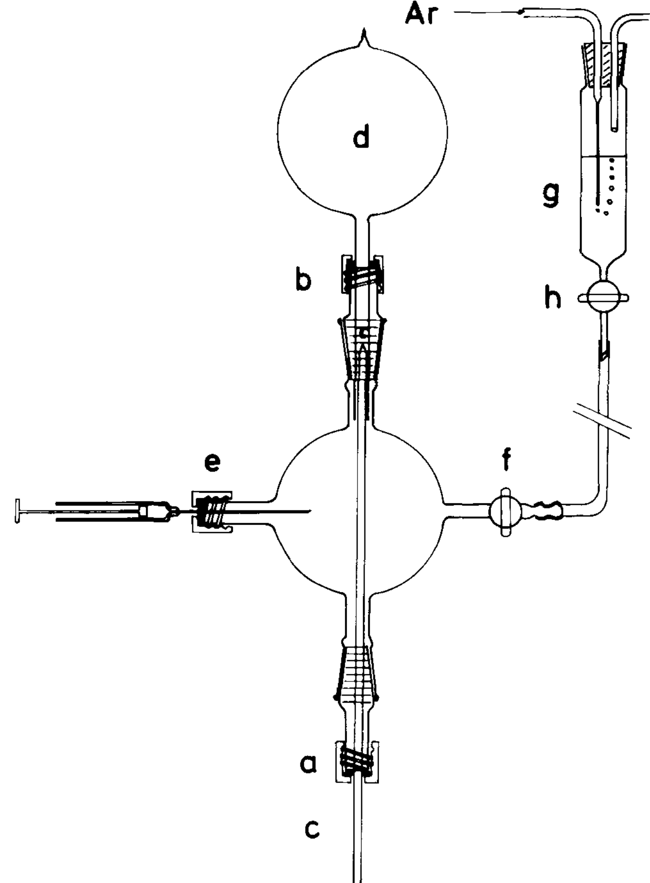

FIG. 1. Vessel for preparation of 13-[$^{18}O_2$]hydroperoxy 9,11-octadecadienoic acid by lipoxygenase-catalyzed oxygenation of linoleic acid with $^{18}O_2$. Description see experimental part.

borate buffer corresponding to the volume of the [$^{18}O_2$] ampoule; both stop-cocks were then left open. Soybean lipoxygenase I (2 mg in 50 μl borate buffer) was injected through the adapter (e). The seal of the ampoule was broken by pushing the glass rod in; the rod was pulled back, and the suspension of linoleic acid and the oxygen gas were equally distributed between vessel and ampoule by turning and shaking the whole apparatus. Every 15 *min, 2 mg* lipoxygenase were added and each time the contents were thoroughly mixed. The progress of the reaction was checked by UV analysis at 235 nm. The reaction was complete after about 90 min. Aqueous citric acid (3%, 20 ml) was used to fill the dropping funnel and 60 ml was injected through the septum (e). Argon was introduced through a needle inserted into the septum. During this procedure the vessel was positioned as shown in Figure 1, with the ampoule pulled back as far as possible. Argon was admitted to make up for the volume of consumed oxygen. The ampoule could then be resealed to save most of the residual $^{18}O_2$, however diluted.

The reaction mixture was extracted three times with 50 ml of ether. The combined extracts were dried over anhydrous sodium sulfate, concentrated on a rotary evaporator to about 10 ml and transferred to a silica gel column (200 mm × 8 mm, Si-60). The hydroperoxide was eluted with toluene/methanol/acetic acid (98.7:1:0.3,

v/v/v). The fractions with elution volumes between 100 and 130 ml were combined, diluted with methanol and concentrated under reduced pressure at a temperature of 15 C. Toluene and acetic acid were removed by repeated azeotropic distillation with methanol. The [$^{18}O_2$]hydroperoxy octadecadienoic acid was stored in methanolic solution at −18 C. A small aliquot was reduced, derivatized and analyzed by GC/MS as described below, showing an isotopic purity of 91% and an isomeric composition of 95% 13-isomer and 5% 9-isomer. The yield, based upon UV analysis, was 32%.

[^{18}O]Hydroxy octadecadienoic acid. [^{18}O]Hydroxy octadecadienoic acid was prepared by reduction of [$^{18}O_2$]hydroperoxide (30). Sodium borohydride was preferred over other reducing agents; triphenylphosphine oxide, for instance, was more difficult to remove during chromatographic purification.

Hydroxy octadecanoic [$^2H_3$]methyl esters. Hydroxy octadecanoic acid, obtained from a commercial supplier and containing all isomers hydroxylated between the 6- and 15-positions, was esterified by heating to 110 C with hydrogen chloride (3 mol/l) in [$^2H_4$]methanol. After 30 min, excess reagent was evaporated under a stream of nitrogen, the residue was dried in vacuo over potassium hydroxide and dissolved in hexane/isopropanol (99.75:0.25, v/v). The isomers were separated by micropreparative high performance liquid chromatography (HPLC) (31) on a silica gel column (25 cm × 0.4 mm, Lichrosorb Si 60, 10 μm, Merck) with hexane/isopropanol (99.75:0.25, v/v) as mobile phase at a flow rate of 1.5 ml/min. Fractions containing the 9-, 10-, 12- and 13-hydroxy octadecanoic [$^2H_3$]methyl esters were collected. The relative and absolute amounts were determined by gas chromatography on a glass capillary (25 m × 0.25 mm) deactivated by silanization (32) and coated with OV-101, film thickness 0.1 μm, and UV analysis at 206 nm.

Quantitative determination of 13-hydroperoxy and 13-hydroxy 9,11-octadecadienoic acid: sample preparation and derivatization. For determination of 13-hydroperoxy and 13-hydroxy 9,11-octadecadienoic acid formed upon aerobic incubation of microsomes with Fe^{2+}/ADP, 3 μg of [$^{18}O_2$]hydroperoxy octadecadienoic acid/mg microsomal protein were added as internal standard. When samples from untreated animals or from animals exposed to tetrachloromethane were analyzed, 50 ng/mg protein was added to microsomes and 10 ng/mg protein to liver homogenate. For determination of 13-hydroxy 9,11-octadecadienoic acid, [^{18}O]hydroxy octadecadienoic acid served as internal standard.

Triphenylphosphine, 200 μg in 10 ml ether per mg protein, was added, and the mixture was stirred at 0 C; reduction was omitted when hydroxy acid alone was to be determined. After 1 hr, the ether was evaporated, the residue was dissolved in 15 ml chloroform/methanol (2:1, v/v) and the solution was filtered through a glass-fiber filter (No. 6, Schleicher and Schuell, FRG). The volume was reduced to 0.5 ml on a rotary evaporator, and the concentrated extract was transferred to a culture tube (10 cm × 1 cm). Phospholipase A_2—20 μg in 1 ml borate buffer, 125 mmol/l, pH 9.0, containing 0.1 mg calcium chloride—was added and the mixture was shaken vigorously. After 90 min, it was adjusted to pH 5 with aqueous citric acid (3%) and the ether layer was transferred to another culture tube and brought to dryness under a stream of nitrogen. The residue was dissolved in 1 ml ether/methanol (2:1, v/v), and diazomethane was bubbled into the solution for 5 min. After 30 min at room temperature, the sample was brought to dryness and the residue dissolved in 25 μl of *bis*-trimethylsilyltrifluoroacetamide, 25 μl pyridine and 10 μl trimethylchlorosilane. The sample was ready for GC/MS after 20 min at room temperature.

Determination of positional isomers. For determination of the isomeric composition, the extracted lipids were reduced with triphenylphosphine and treated with phospholipase A_2. PtO_2-catalyzed hydrogenation yielded the corresponding hydroxy octadecanoic acid isomers, which were esterified with diazomethane. The 9-, 10-, 12- and 13-isomers of hydroxy octadecanoic [$^2H_3$]methyl ester were added as internal standards in amounts as expected to be present in the sample. After trimethylsilylation, the samples are analyzed by GC/MS.

GC/MS. The instrument is a Finnigan 4021 with an Incos data system. For GC, aliquots containing 1–50 ng hydroxy fatty acid derivatives were injected onto a silanized glass capillary coated with OV-101. The injector temperature was 280 C; the silanized glass insert of the injection port was replaced after every five injections. Injections were done in the splitless mode at an initial oven temperature of 60 C. Half a minute later, the split valve was opened and the following temperature program was started: 0.5 min isothermal, 40 C/min to 180 C, 4 C/min to 300 C. The carrier gas was helium with an inlet pressure of 100 kPa. The GC/MS interface was a silanized fused-silica capillary (40 cm × 0.1 mm). The interface temperature was 300 C.

For MS, the ion source temperature was 290 C, ionizing voltage was 35 eV, scan speed from m/z 50 to 550 was one second and secondary electron multiplier voltage was 1.6 kV. When analyzing in vitro samples, the mass spectrometer was scanned over the total mass range and reconstructed mass fragmentograms were generated; for in vivo samples, multiple ion detection was preferred.

RESULTS AND DISCUSSION

Tetrachloromethane is thought to cause lipid peroxidation by hydrogen abstraction from polyunsaturated fatty acids. This is initiated by trichloromethyl radicals that are formed via cytochrome P-450-dependent reduction (33,34). For other xenobiotics, generation of hydroxyl radicals has been invoked (35). The next stage in this hypothetical sequence is addition of oxygen to yield peroxy radicals and, by abstraction of hydrogen atoms from other polyunsaturated fatty acids, monohydroperoxides.

Monohydroperoxides of linoleic and arachidonic acid are detectable in rat liver microsomes after aerobic incubation with Fe^{2+}/ADP (20). Hydroperoxides derived from linoleic acid are present in highest concentrations; the 9-, 10-, 12- and 13-isomers have been found. Those of arachidonic and docosahexaenoic acid are present in much smaller amounts. It is likely that the latter undergo secondary reactions faster than the linoleic acid derivatives due to their greater number of double bonds. We therefore concentrated upon determination of the linoleic acid derivatives.

The method used for determination of 13-hydroperoxy and 13-hydroxy 9,11-octadecadienoic acid in microsomal lipids after in vitro and in vivo experiments involves

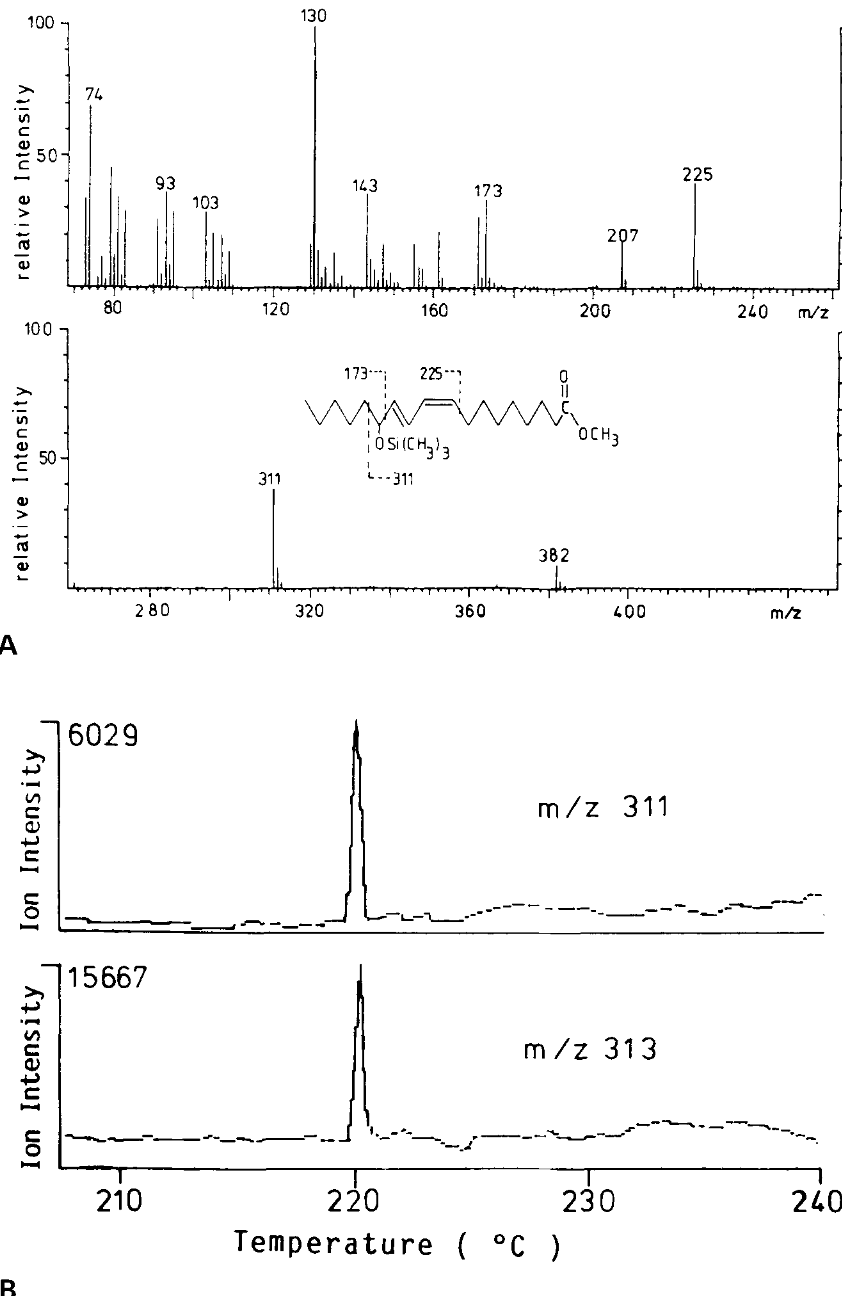

FIG. 2. (a) Mass spectrum of 13-trimethylsiloxy 9,11-octadecadienoic methyl ester; (b) selected ion-current profiles of m/z 311 and m/z 313 (internal standard) in microsomes from a control animal.

internal standardization with [$^{18}O_2$]hydroperoxy 9,11-octadecadienoic acid, reduction to the corresponding hydroxy fatty acid and derivatization to yield 13-trimethylsiloxy 9,11-octadecadienoic acid methyl ester. The samples are analyzed by gas chromatography and mass spectrometry monitoring the abundant α-fragments, m/z 311 and 225 for the endogenous hydroperoxide and m/z 313 and 227 for the ^{18}O-labeled standard (Fig. 2). It is important to verify the respective retention times with authentic standards, as several oxygen-containing derivatives of arachidonic or docosahexaenoic acid yield ions with the same masses. The quantity of 13-hydroperoxy plus 13-hydroxy 9,11-octadecadienoic acid is calculated from the ratios of the peak areas of the ion chromatograms of m/z 311 vs 313 and 225 vs 227, taking the percentages of isotopic and isomeric purity of the internal standard into account.

Isotopic dilution analysis with two different internal standards, i.e., [$^{18}O_2$]hydroperoxy and [^{18}O]hydroxy fatty acid, allows differential analysis for hydroperoxy and hydroxy fatty acids. The sum of hydroperoxy and hydroxy fatty acids is determined with [$^{18}O_2$]hydroperoxy

TABLE 1

Amount of 13-Hydroperoxy Plus 13-Hydroxy 9,11-octadecadienoic acid [HO] Determined by Isotopic Dilution Analysis in 1 ml of Rat Liver Homogenate (33.6 mg protein/ml) with Different Amounts of [$^{18}O_2$-IS] (a) or [^{18}O-IS] (b) as Internal Standard and [$^{16}O_2$-HO] Spiked (c).

	$^{18}O_2$-IS (nmol)	^{18}O-IS (nmol)	$^{16}O_2$-HO spiked (nmol)	HO found (nmol)	HO (pmol/mg protein)	Mean ± S.D. (pmol/mg protein)
(a)	3.00	—	—	0.28	8.3	
	1.50	—	—	0.41	12.2	
	0.75	—	—	0.36	10.7	10.7 ± 1.7
	0.38	—	—	0.40	11.9	
(b)	—	1.50	—	0.34	11.5	
	—	3.00	—	0.44	13.2	13.3 ± 1.8
	—	3.00	—	0.54	15.2	
(c)	6.08	—	6.76	7.27	15.2[b]	
	6.08	—	3.38	3.77	11.6[b]	
	6.08	—	1.69	2.15	13.7[b]	14.0 ± 1.8
	6.08	—	0.68	1.20	15.5[b]	

[a][$^{18}O_2$-IS], 13-[$^{18}O_2$]hydroperoxy 9,11-octadecadienoic acid; [^{18}O-IS], 13-[$^{18}O_2$]hydroxy 9,11-octadecadienoic acid; [$^{16}O_2$-HO] spiked, nonlabeled synthetic 13-hydroperoxide. In series (b), triphenylphosphine reduction has been omitted.
[b]Values calculated after subtraction of spiked amounts of 13-hydroperoxy 9,11-octadecadienoic acid.

TABLE 2

13-Hydroperoxy 9,11-octadecadienoic Acid and 13-Hydroxy 9,11-octadecadienoic Acid in Rat Liver Microsomes After Typical Fe^{2+}/ADP-induced Lipid Peroxidations

13-Hydroperoxy + 13-hydroxy 9,11-octadecadienoic acid (n mol/mg protein)	13-Hydroxy 9,11-octadecadienoic acid (n mol/mg protein)	13-Hydroperoxy 9,11-octadecadienoic acid (n mol/mg protein)
7.9	1.0	6.9
6.7	0.7	6.0
5.4	0.7	4.7
7.9	1.4	6.6
6.7	1.1	5.6
$\bar{x}$ ± S.D. = 6.9 ± 1.1	$\bar{x}$ ± S.D. = 1.0 ± 0.3	$\bar{x}$ ± S.D. = 6.0 ± 0.9

fatty acid as internal standard. A second aliquot with [^{18}O]hydroxy fatty acid as internal standard serves for determination of hydroxy fatty acid alone. The difference between both represents hydroperoxy fatty acid.

Internal standards labeled with ^{18}O in the hydroperoxy moiety have been used to study the rearrangement of linoleic acid hydroperoxides (36). Hydroperoxy fatty acids ^{18}O-labeled in the carboxyl group (37) are not suitable, since the internal standard should not only serve for correction of volumetric errors and as reference for calculation of response factors, but also for compensation of chemical losses. Also, ^{18}O-labeling of the carboxyl group is difficult to achieve with high isotopic yield, and under our conditions of sample pretreatment and derivatization, exchange of oxygen took place.

Nonspecific formation of hydroxy fatty acids from hydroperoxides may falsify the results from samples not reduced with triphenylphosphine. Hydroperoxides may nonspecifically form hydroxy acids, e.g., via reactions as proposed by Russel (38). Its significance was investigated by adding increasing amounts of synthetic nonlabeled 13-hydroperoxy 9,11-octadecadienoic acid to aliquots of a microsomal sample. Nonspecific conversion of about 5% of added hydroperoxide to the corresponding hydroxide took place. This was accounted for when endogenous hydroxy fatty acids were determined in nonreduced samples.

The accuracy of the method was tested with liver homogenate from untreated Sprague-Dawley rats. In a first series of analyses (Table 1, series a), increasing amounts of 13-[$^{18}O_2$]hydroperoxy 9,11-octadecadienoic acid were added. The precision of determination was about ±15%, sufficient to monitor the trends in the following in vitro and in vivo experiments. In a second series (Table 1, series b), reduction of the samples with triphenylphosphine was omitted to determine 13-hydroxy 9,11-octadecadienoic acid alone; the quantity found was not different from the values obtained in series a, showing that all of linoleic acid oxidized in position 13 was present as hydroxide. Its level in microsomes from untreated animals was in the range of 10 to 14 pmol/mg protein. Whether this was an in vivo constituent or an artifact formed during preparation cannot be decided.

In one series (not shown), samples were spiked with synthetic nonlabeled 13-hydroperoxide and kept at 0 C

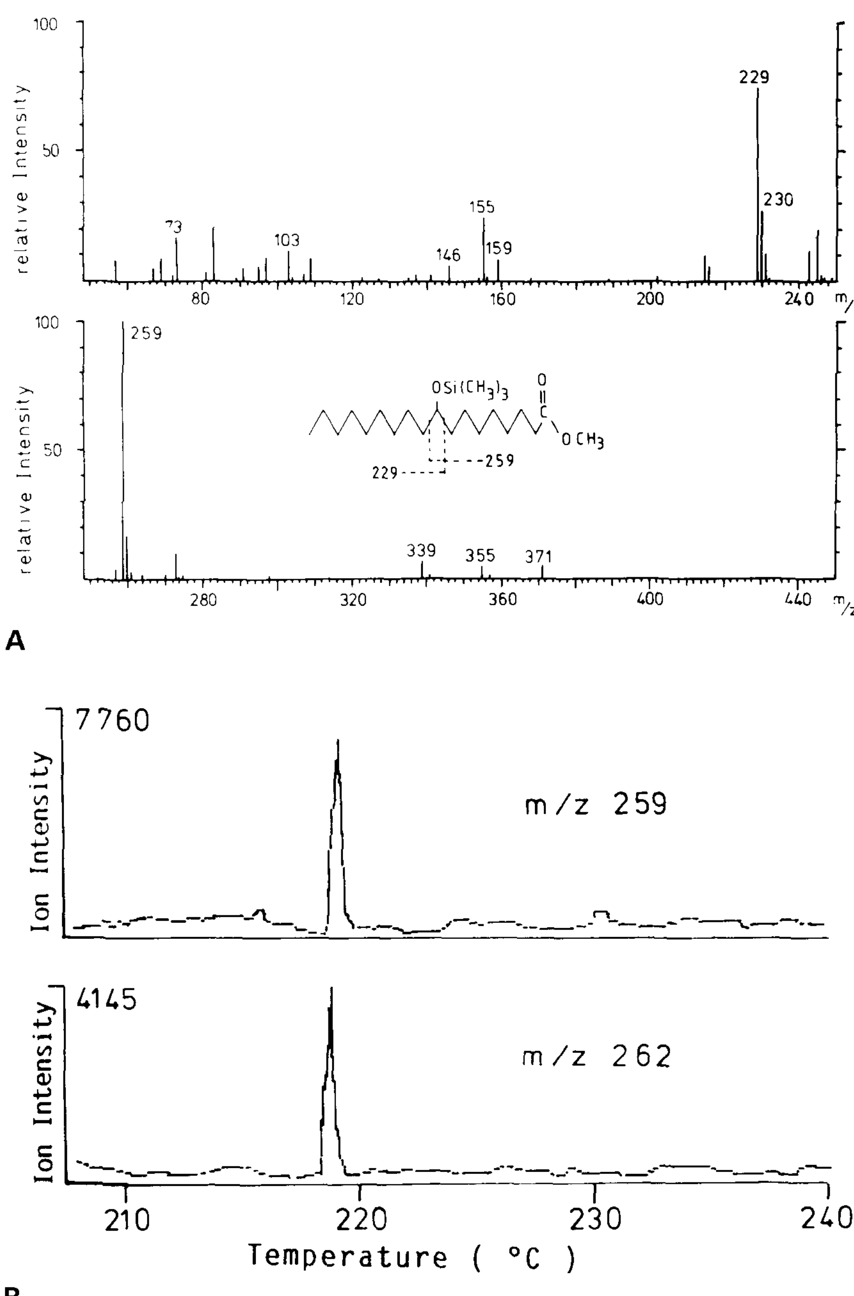

FIG. 3. (a) Mass spectrum of 9-trimethylsiloxy octadecanoic methyl ester; (b) selected ion-current profiles of a sample from an aerobic microsomal incubation with Fe^{2+}/ADP; m/z 259 = endogenous 9-hydroxy octadecanoic methyl ester; m/z 262 = $^{2}H_3$-labeled internal standard.

for 1 hr before the ^{18}O-labeled internal standard was added. In this case, only 24% of the spiked amount was recovered, showing the importance of rapid work-up.

The amounts of 13-hydroperoxy and 13-hydroxy 9,11-octadecadienoic acid found after 15 min of aerobic incubation of microsomes with Fe^{2+}/ADP are shown in Table 2. Hydroperoxide was formed in large amounts; apparently, some of it was reduced to the hydroxide.

For determination of the relative amounts of the isomers of oxidized octadecadienoic acid, they were hydrogenated to the hydroxy octadecanoic acid isomers. The 9-, 10-, 12- and 13-hydroxy octadecanoic [$^{2}H_3$]methyl esters were added as internal standards. Quantitative determination was done by GC/MS monitoring the C-terminal α-fragments (Fig. 3, Table 3). The isomeric composition after aerobic incubation of microsomes with Fe^{2+}/ADP is shown in the last column of Table 3. The relative values are in good agreement with previous results (20). The 9-isomer predominated, in contrast to the even isomer distribution after homogeneous autoxidation (31). The reason for this peculiar pattern is unknown. Small amounts of 10- and 12-isomers were found, which are probably

TABLE 3

Fragment Ions in the EI Mass Spectra of 9-, 10-, 12- and 13-Trimethylsiloxy octadecanoic Methyl Esters, Resulting from Cleavage of the Bond of the Trimethylsiloxy-carbon Proximal (α_ω) or Distal (α_c) to the Carboxyl Terminal. In the second column (α_c) the lower number of each row corresponds to the $[^2H_3]$-methyl ester. The last column shows the relative percentage of each isomer found after aerobic incubation of microsomes with Fe^{2+}/ADP (n = 4).

Isomer	α_ω (m/z)	α_c (m/z)	Relative amount in % (mean ± S.D.)
9	229 (75)	259 262 (100)	56 ± 2
10	215 (100)	273 276 (85)	5 ± 2
12	187 (100)	301 304 (30)	5 ± 2
13	173 (100)	315 318 (60)	33 ± 3

Relative abundances indicated in parentheses.

"ene" reaction products of singlet oxygen. No hydroxy octadecanoic acids other than the 9-, 10-, 12- and 13-isomers were detected.

The absolute values determined previously without internal standardization have been about 10-fold lower (20). Obviously, when the decomposition of hydroperoxides during sample preparation is not accounted for, reliable quantitative determination is not feasible.

The amounts of 13-hydroperoxy and 13-hydroxy 9,11-octadecadienoic acid in liver homogenate and microsomes from untreated rats and animals exposed to tetrachloromethane are shown in Table 4. The sum of 13-hydroperoxide and 13-hydroxide found in liver homogenate or microsomes of control animals was similar to the contents in 13-hydroxide. Consequently, only very little can be present as hydroperoxide. Treatment of rats with 75 mg tetrachloromethane/kg body weight, which causes massive centrilobular necrosis (39), entailed a small, insignificant increase of the sum of hydroperoxide and hydroxide in the homogenate. In microsomes, a significant increase of the sum of hydroperoxide and hydroxide was found, but not of hydroxide alone. 13-Hydroperoxy 9,11-octadecadienoic acid was increased by about 140 pmol/mg microsomal protein, which is considerably less than after a typical microsomal lipid peroxidation in vitro.

In comparison to the values reported for hydroxy-eicosatetraenoic acid isomers, which have been obtained without internal standardization, and not differentiating between hydroperoxy and hydroxy derivatives (40), the standard deviations for hydroxy-octadecadienoic acid isomers were considerably larger between individual animals, in spite of the small relative standard deviation of the analytical method (15%). In contrast to hydroxy-eicosatetraenoic acid (40), no significant increase of oxidized octadecadienoic acid was found in liver homogenate from rats treated with tetrachloromethane. It is not known whether this may be due to the much larger dose of tetrachloromethane administered in that study (3.2 g/kg). However, the effective dose is difficult to assess when the animals are kept in open cages, as most of it is exhaled unchanged (41).

The low levels of hydroperoxides found after tetrachloromethane exposure in vivo suggest that either formation of oxidized fatty acids was slow or their detoxification efficient. Preliminary experiments showed that the amounts of oxidized linoleic acid increased during exposure to tetrachloromethane in the course of several hours. Apparently, not only the extent but also the rate of oxidation is different between aerobic microsomal incubations and tetrachloromethane exposure in vivo. While in the former case the critical reactions took place within a quarter of an hour, metabolism of tetrachloromethane in vivo and the consecutive fatty acid oxidation proceeded within several hours.

Admittedly, additional aspects must be addressed before definite conclusions can be drawn. Thus, it must be considered that the liver tissue is damaged by tetrachloromethane in a focal pattern, and that the procedures employed for isolation of microsomes do not discriminate between affected and nonaffected regions. Also, the labeled fatty acid hydroperoxides employed as internal standards may differ in their chemical and biochemical properties from phospholipid hydroperoxides. Therefore, the absolute values determined by the method presented here may still deviate from the true values. Nevertheless, for a comparative study as undertaken here, only relative values are needed.

TABLE 4

13-Hydroperoxy 9,11-octadecadienoic Acid and 13-Hydroxy 9,11-octadecadienoic Acid in Liver Homogenate and Microsomes from Untreated and Tetrachloromethane-exposed Male Sprague-Dawley Rats (means ± S.D., n = 3)

	13-Hydroperoxide + hydroxide (pmol/mg protein)	13-Hydroxide (pmol/mg protein)
Controls		
Homogenate	19 ± 7	20 ± 7
Microsomes	87 ± 28	102 ± 33
CCl_4-treated		
Homogenate	27 ± 10	17 ± 9
Microsomes	230 ± 40	118 ± 17

Microsomal lipid peroxidation, induced by hydrogen peroxide-producing enzymes (42) in the presence of iron and oxygen in high concentrations, does not parallel the formation of oxidized fatty acids elicited in vivo by tetrachloromethane. This must be viewed in context with other evidence. Several cellular defense lines can prevent lipoperoxidative damage to membranes, which may explain why in vivo the levels of hydroperoxides are low. Enzymes scavenging reactive oxygen species and other protective mechanisms are operative in vivo. It is known that oxygen-modified fatty acid residues in phospholipids are rapidly excised by phospholipases (43) and/or are reduced to hydroxy fatty acids (44,45). Glutathione-dependent processes and various antioxidants afford further protection against oxidative damage (46,47).

It is likely that autoxidation proceeds relatively rapidly when all these mechanisms are absent, as is typical for aerobic microsomal incubations. However, the complex question about involvement or significance of lipid peroxidation in pathogenicity in vivo is not a qualitative one, but rather needs to be answered in quantitative terms. From this point of view, it is questionable whether in vitro experiments involving massive oxidative breakdown of polyunsaturated fatty acids, sometimes under addition of nonbiological hydroperoxides and performed in the absence of all cellular defense systems that are usually operative, may yield useful information for understanding the processes involved in vivo. The significance of lipid peroxidation must be reconsidered, and a clearer definition of the term is necessary. Further development of suitable analytical methods is required to clarify whether it is more than an in vitro phenomenon.

The results lend little support to the view that peroxidative processes are causally related to tetrachloromethane toxicity. Other effects such as covalent modification and cross-linking of polyunsaturated fatty acids (48) and the ensuing inactivation of anabolic enzymes of the endoplasmic reticulum are probably more significant as primary lesions (49).

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Lipid Composition of *Bacillus megaterium* Spores and Spore Membranes

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***Bacillus megaterium* QM B1551 spore lipids were extracted by an improved technique, and the phospholipid and fatty acid compositions were determined. Phospholipids accounted for 65% of the total fatty acids; the neutral lipid fraction contained 15% and the remaining fatty acids were in the interphase, aqueous phase and pellet from the lipid extraction. Each phospholipid had similar fatty acid compositions as did the delipidated pellet. However, the aqueous phase and, to some extent, the interphase had unique fatty acid compositions. Also, fatty acids were found acylated to proteins, which was observed by electrophoresis of delipidated proteins from spores grown in [1-^{14}C]palmitate. Therefore, spores contain unique non-phosphatide fatty acid components that can now be analyzed.**
Lipids 22, 698-703 (1987).

Bacilli are relatively unique in containing a large proportion of branched chain fatty acids (1). We and others have determined the fatty acid and phospholipid composition of *Bacillus megaterium* QM B1551 spores (2-4). Curiously, about 50% of the spore fatty acids have been reported to be in a non-phosphatide fraction (3). In an attempt to better define the lipid composition of spores, we have found that previous measurements have significantly underestimated both the fatty acid and phospholipid contents. In addition, we found previously unreported fatty acids, another phospholipid and diacylglycerols. We attribute these new findings to better lipid extractions and improved chromatographic procedures.

These data are important because they provided the basis for our identification of a unique class of phospholipids associated with protein in spore membranes (Nikolopoulou, M., and Vary, J.C., in preparation), the occurrence of spore proteins with covalently bound fatty acids (reported here) and the presence of diacylglycerol that stimulates a protein kinase for protein phosphorylation during sporulation (unpublished).

MATERIALS AND METHODS

Materials. Organic solvents were ACS grade or better from Fisher Scientific (Chicago, IL). Fatty acid standards were from Supelco Inc. (Bellefonte, PA). Silica Gel H was from E.M. Reagents (EM Laboratories, Cincinnati, OH), and other chemicals were from Sigma Chemical Co. (St. Louis, MO) or CalBiochem (La Jolla, CA). Radioactive precursors were from ICN Biomedicals (Irvine, CA).

Preparation of spores and homogenates. Spores of *B. megaterium* QM B1551 were grown in supplemented nutrient broth, washed and extracted with SDS-DTT to make them lysozyme-sensitive (5,6). Spore homogenates and membranes were prepared by lysis and sonication as previously described (7,8). In some cases, spores were grown with [1-^{14}C]palmitate (sp act 58 mCi/mmol), which was added to late exponential phase cells at a final concentration of 10 μCi/ml. All references to spore weights are on a dry weight basis.

Lipids were extracted from disrupted spores by two methods. In method A, spores were extracted with SPS as described by Bligh and Dyer (9) and were modified (2). In method B, spores were incubated in SPS at room temperature for 1 hr with periodic vortexing. The suspension was centrifuged at 6,000 × g for 10 min, and the pellet was extracted four more times with 10 min of incubation in SPS and vigorous vortexing. The combined supernatant fractions were separated into aqueous and organic solvent phases as described (2). The lower organic phase was collected, and the aqueous phase extracted four more times with fresh lower phase. The organic fractions were combined, washed once with 20% volume of fresh upper phase, dried under N_2 and resuspended in $CHCl_3$/methanol (2:1, v/v). The aqueous phase was centrifuged once at 6,000 × g for 5 min, and the supernatant fraction was separated from the white interphase. Finally, the interphase was dissolved in $CHCl_3$/methanol (2:1, v/v).

Phospholipids were separated by TLC on Silica Gel H plates that were prerun in methanol and activated at 110 C for 30 min. The solvent was chloroform/methanol/acetic acid/H_2O (60:20:8:0.5, v/v/v/v), and spots were visualized by I_2 vapor, molybdate spray or autoradiography on Kodak X-Omat AR film. Quantitation was by scraping and phosphate assay (10) or by measuring radioactivity (5).

To determine the fatty acid composition of different lipid fractions, 2 ml of 4% sulfuric acid in methanol and 50 nmol of nonadecanoic acid were added, and the samples were transesterified for 60 min at 70 C. Upon cooling, 1.0 ml of H_2O was added, and the methyl esters were extracted with 2 ml of petroleum ether. Total spore fatty acids were determined by direct transesterification of intact spores (20 mg) in 2.0 ml of 4% sulfuric acid in methanol. Strong acid is known to completely disrupt spores, called "acid popping" (11,12), making all fractions of the spore accessible to methylation. To separate the fatty acid methyl esters from any contaminants, the petroleum ether extract was applied on activated Silica Gel H plates and chromatographed in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). After development, the area corresponding to standard methyl esters (R_f = 0.7) was scraped and eluted with petroleum ether. The petroleum ether extract was dried with a stream of N_2, and the methyl ester residue was dissolved in 20-30 μl of distilled CS_2 and analyzed by GLC.

Methyl esters were separated on a Varian model 3700 chromatograph equipped with a 2 m × 2 mm id stainless steel column packed with GP 10% DEGS-PS on 80/100 Supelcoport. The column oven was programmed to maintain 135 C for 10 min, then to increase to 160 C at 3 C/min and finally to maintain at 160 C for 10 min. The

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Abbreviations: DTT, dithiothreitol; GLC, gas liquid chromatography; SDS-DTT, sodium dodecylsulfate-dithiothreitol; SPS, single phase solvent; TLC, thin layer chromatography.

injection port and the detector were at 200 C and 250 C, respectively. The carrier gas was N_2 at a flow rate of 30 ml/min.

Quantitation of the fatty acids in each sample was achieved by comparing the peak areas relative to the standard nonadecanoic acid with the aid of an integrator. The fatty acid methyl esters were identified by comparing their equivalent chain length with standards and were confirmed by mass spectrometry on a Finnigan 4510 spectrometer equipped with a 30 m × 0.25 mm id fused silica capillary column coated with SP-2330. The spectrometer was operated with electron multiplier at 70 kV, emission current −0.25 mA and an electron energy of 70 eV. The carrier gas was helium at a pressure of 22 psi.

In all cases, blanks (no sample) were run through the same extraction procedures and analyzed by GLC, as were all solvents. The experiments reported here had blanks close to background, and all values were corrected for the blanks. Also, all glassware used for lipid analyses was rinsed with methanol. By using these precautions and methylation procedures, we discovered an artifact in our previously published fatty acid compositions (4). The diethyl ether used previously contained an antioxidant, butylated hydroxytoluene, which appeared as n-C14 by retention time in GLC. Therefore, the values for fatty acids reported here represent a correction and, as noted above, the identity of each fatty acid was confirmed by mass spectrometry.

Electrophoresis. The disrupted spores were delipidated as described above and electrophoresed in 13% polyacrylamide gels by the method of O'Farrell (13), except no dithiothreitol (DTT) was added unless indicated. After staining and destaining, the gels were enhanced and subjected to fluorography. Hydroxylamine treatment of delipidated protein before or after electrophoresis was performed exactly as described previously (14).

RESULTS AND DISCUSSION

Fatty acid content. The total fatty acid content of *B. megaterium* spores was determined by direct transesterification of intact spores. For one batch of spores, 44.4 µmol of fatty acids/g of spores was found, and this same batch was used for all of the studies described below. Other spore batches had different values, with an average of 48 ± 10 µmol/g of spores (± standard deviation, n = 5), which was higher than the value of 39 that we calculated from previous results (3). However, we wish to note that the absolute values are probably not significant and direct comparisons between the fatty acid contents of different spore batches should not be made. Not only were different spore batches variable, but different lots of nutrient broth produced spores with different total fatty acid contents. Finally, it may be noted that SDS-DTT "coat-stripped" spores were used in all studies, but when nonstripped spores were used, the fatty acid content was 53 ± 6 µmol/g of spore (± standard deviation, n = 3), which shows that within experimental error, SDS-DTT extracted and nonextracted spores had the same fatty acid content. This last observation agrees with our previous results on the similarity of phospholipid content between extracted and nonextracted spores, suggesting that it is unlikely that these spores contain an intact functional outer membrane (7), which would have been removed by SDS-DTT.

Lipid extraction. To investigate the lipids, we mechanically disrupted the *B. megaterium* spores and extracted the lipids by method A of Bligh and Dyer, which has been routinely used (2,4,7,15) for analysis of bacillus lipids. Only 68% of the total spore fatty acids were in the organic phase of the lipid extract (Table 1), and the remainder was distributed in other fractions. Method B recovered an additional 5.2 µmol of fatty acid in the organic phase of the lipid extract, which came mainly from the pellet, since the interphase and aqueous phase remained about the same. Further extractions of the delipidated pellets failed to recover any significant additional amounts of fatty acid (<1%). Note that the sum of the fatty acid contents of the various lipid fractions was the same as found by transesterification of whole spores.

Similarly, from spore homogenates, methods A and B extracted 11 ± 1 and 14 ± 2 µmol of phospholipid phosphate/g of spore (± standard deviation, n = 4), respectively. Several other methods of extraction were tested exactly as described by Cohen et al. (16), Folch and Lees (17) and Rose and Oklander (18), and all gave values that ranged from 10 to 13 µmol of phospholipid phosphate/g of spore.

Fatty acids. The fatty acid composition of each fraction in Table 1 was determined by GLC, and the identity of each fatty acid was assigned by retention time and confirmed by mass spectrometry (Table 2). For total spore fatty acids, the major species were i-C14, i-C15 and a-C15, which represented almost 80% of all species. The C18 species were previously not reported because C17 or C18 was used as an internal standard (3,4); in a separate experiment, we determined that spores had no C19 fatty acids, which is why we used that as our internal standard.

When the lipids were extracted from spore homogenates, the fatty acid composition of the organic phase was similar to that of the whole spores, except for more i-C16 and less i-C15 and a-C15 in the former. However, the interphase was enriched for all C16 and C18 species, and the aqueous phase was enriched for i-C16 and i-C18. The delipidated pellet was similar (within experimental error) to the whole spores and probably represents unbroken spores.

TABLE 1

Fatty Acid Content of Spore Homogenate Lipid Extract[a]

	Extractable fatty acids/g of spore			
	Method A		Method B	
Fraction	µmol	%	µmol	%
Organic phase	30.0	68	35.2	80
Interphase	3.5	8	2.6	6
Aqueous phase	4.0	9	3.5	8
Delipidated pellet	6.8	15	2.7	6
Total	44.3	100	44.0	100

[a]The fatty acid content of single phase solvent-extracted spore homogenates was determined as described in the text. Values are from one batch of spores which, by direct transesterification of whole spores, contained 44.4 µmol of fatty acid/g of spore.

TABLE 2

Fatty Acid Composition of the Spore Fractions[a]

Fatty acid	Whole spores	Organic phase	Interphase	Aqueous phase	Delipidated pellet
i-C14	17.3 ± 0.4	19.9 ± 2.0	19.7 ± 4.5	11.8 ± 2.8	14.1 ± 1.1
n-C14	1.9 ± 0.7	1.7 ± 0.4	3.1 ± 2.2	1.2 ± 0.7	1.7 ± 0.3
i-C15	26.1 ± 0.5	21.8 ± 1.3	14.4 ± 2.0	12.4 ± 2.3	24.3 ± 2.7
a-C15	35.2 ± 0.5	29.7 ± 1.6	19.1 ± 2.9	14.9 ± 3.2	32.7 ± 3.5
n-C15	1.9 ± 0.2	1.4 ± 0.2	1.1 ± 1.1	0.9 ± 0.6	1.5 ± 0.3
i-C16	4.0 ± 0.3	10.2 ± 0.5	13.2 ± 2.3	32.5 ± 4.9	4.3 ± 0.3
n-C16	4.0 ± 1.6	3.9 ± 0.4	8.4 ± 1.9	5.5 ± 1.3	5.4 ± 1.1
i-C17	1.2 ± 0.8	2.0 ± 0.1	—[b]	2.4 ± 2.0	3.1 ± 1.7
a-C17	3.4 ± 0.6	2.7 ± 1.3	—	1.1 ± 0.8	3.1 ± 0.5
i-C18	1.3 ± 0.5	3.5 ± 0.6	9.5 ± 0.8	15.2 ± 2.0	3.7 ± 2.5
n-C18	1.8 ± 0.7	2.1 ± 1.2	6.6 ± 1.3	1.3 ± 0.7	2.7 ± 1.1
n-C18:1	1.9 ± 0.6	1.1 ± 0.3	5.0 ± 1.6	0.9 ± 0.4	3.4 ± 0.3

[a]The fatty acid composition of each fraction from Table 1 was determined as described in the text. Values are mol % representing the mean ± standard deviations, n = 4.
[b]Not detectable.

TABLE 3

Phospholipid Composition[a]

Phospholipid	Homogenate	Membranes
Phosphatidylglycerol	36.7 ± 2.7	42.1 ± 3.5
Diphosphatidylglycerol	26.8 ± 2.8	20.2 ± 1.1
Phosphatidylethanolamine	17.2 ± 2.6	16.5 ± 1.0
Glucosaminylphosphatidylglycerol	14.5 ± 0.6	14.9 ± 0.9
X (unidentified phospholipid)	4.7 ± 1.0	6.3 ± 2.3

[a]Spore homogenates or membranes were extracted by method B, and the organic phases were separated by thin layer chromatography as described in the text. Values are mol % representing the mean ± standard deviations, n = 4 for homogenates and n = 3 for membranes.

Phospholipids. The organic phases of the lipid extract from spore homogenates and, for comparison, spore membranes were separated by TLC and quantitated by phosphate assays. The results (Table 3) show a distribution similar to previous data (2,3) with the exception of an additional minor phospholipid "X" that has not been identified. This phospholipid X was not seen by previous investigators because their TLC solvent system did not separate X from diphosphatidylglycerol (DPG). The possibility of any other unknown phospholipids was investigated by two-dimensional TLC of lipid extracts from $^{32}PO_4$-grown spores as previously described (19). In addition to the five spots reported here, there were some barely detectable spots representing common phospholipid metabolic intermediates like phosphatidic acid and CDP-diacylglycerol, but these represented less than 1% of the total. There was no radioactivity where phosphatidylserine or inositol phospholipids would migrate, and we could have detected them at the level of <1% (data not shown). Therefore, all of the lipid phosphate could be accounted for as phospholipid.

Fatty acid composition of phospholipids. The fatty acid composition of each phospholipid class was determined for both homogenates and purified membranes (Table 4). The phospholipids in the homogenate and purified membranes had very similar fatty acid compositions, and the different phospholipids were also similar to each other in fatty acid composition. In all cases, the phospholipids were rich in i-C14, i-C15 and a-C15 like whole spores, and most variations were within experimental error with the exception of a few, e.g., i-C15. There were no phospholipids with unique fatty acid compositions. All fatty acids were linked to the glycerol moieties by ester bonds, since no plasmalogens were detected when assayed by the methods of Owens (20) (data not shown).

Neutral lipids. When lipid extracts (method B) were separated by TLC in hexane/diethyl ether/acetic acid (70:30:1, v/v/v), the phospholipids remained at the origin while free fatty acids and diacylglycerol had approximate R_f values of 0.3 and 0.5, respectively, corresponding to the R_f values of commercial standards. Similar results were found with benzene/diethyl ether/ethanol/acetic acid (50:40:20:0.2, v/v/v/v) as a TLC solvent. The diacylglycerol spot contained two subspecies of 1,2-diacylglycerol and 1,3-diacylglycerol that were not completely resolved and were, therefore, treated as one component. No spots corresponding to monoacylglycerol, triacylglycerol, cholesterol or cholesteryl esters were observed. The fatty acid

TABLE 4

Fatty Acid Composition of Spore Phospholipids[a]

Fatty acid	DPG	PG	GlcNPG	PE	X
		Homogenate			
i-C14	16.1 ± 2.4	18.9 ± 0.5	11.6 ± 2.3	13.9 ± 1.5	19.5 ± 1.4
n-C14	2.5 ± 0.4	2.0 ± 0.5	3.9 ± 1.1	3.9 ± 1.3	4.0 ± 1.7
i-C15	19.1 ± 1.7	24.1 ± 2.3	18.5 ± 1.1	18.1 ± 0.9	15.6 ± 1.7
a-C15	25.2 ± 2.9	30.6 ± 3.1	28.8 ± 2.0	27.7 ± 2.9	19.2 ± 1.5
n-C15	1.8 ± 0.5	1.6 ± 0.3	1.8 ± 0.7	1.5 ± 0.7	2.2 ± 0.7
i-C16	8.7 ± 1.5	3.9 ± 2.6	4.1 ± 0.1	3.5 ± 0.5	8.7 ± 3.2
n-C16	7.3 ± 2.5	5.6 ± 1.5	9.0 ± 1.4	8.5 ± 2.0	11.1 ± 2.8
i-C17	2.5 ± 1.4	3.3 ± 1.5	—[b]	5.4 ± 1.9	3.1 ± 2.4
a-C17	2.7 ± 1.3	2.6 ± 2.0	5.7 ± 3.0	2.9 ± 1.2	—
i-C18	5.6 ± 1.8	2.2 ± 1.3	2.8 ± 1.8	4.6 ± 3.2	5.0 ± 0.8
n-C18	4.3 ± 2.0	2.0 ± 1.0	7.2 ± 4.6	4.8 ± 0.5	5.1 ± 1.7
n-C18:1	4.3 ± 2.0	3.2 ± 2.0	6.5 ± 1.4	5.2 ± 1.0	6.4 ± 1.0
		Membranes			
i-C14	17.7 ± 0.8	18.4 ± 2.6	15.3 ± 2.0	13.1 ± 3.0	13.8 ± 1.8
n-C14	1.5 ± 0.2	1.6 ± 0.3	3.9 ± 0.7	4.1 ± 1.0	1.8 ± 0.3
i-C15	25.6 ± 0.5	23.6 ± 1.3	20.2 ± 1.9	17.0 ± 2.0	19.6 ± 1.7
a-C15	33.1 ± 0.7	30.6 ± 1.6	31.7 ± 2.4	27.1 ± 2.6	25.8 ± 4.9
n-C15	1.6 ± 0.2	1.5 ± 0.3	1.9 ± 0.7	1.4 ± 0.5	3.0 ± 1.7
i-C16	5.4 ± 0.5	5.1 ± 0.9	3.8 ± 0.6	4.9 ± 0.4	3.9 ± 2.6
n-C16	4.3 ± 0.5	4.5 ± 1.0	8.9 ± 1.2	10.8 ± 2.0	9.8 ± 2.9
i-C17	2.0 ± 0.6	2.2 ± 0.5	3.7 ± 2.4	4.7 ± 1.7	2.8 ± 1.8
a-C17	3.6 ± 0.7	3.2 ± 0.6	2.7 ± 1.1	3.4 ± 0.3	2.7 ± 1.1
i-C18	1.9 ± 0.8	3.7 ± 1.9	1.5 ± 0.9	2.8 ± 1.0	1.4 ± 1.6
n-C18	1.6 ± 0.5	2.4 ± 1.0	3.4 ± 0.6	4.9 ± 1.4	9.6 ± 2.7
n-C18:1	1.8 ± 0.6	3.1 ± 1.1	2.9 ± 0.2	5.6 ± 1.3	5.8 ± 2.1

DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GlcNPG, glucosaminylphosphatidylglycerol; PE, phosphatidylethomolamino; X, unidentified phospholipid.
[a]Phospholipids were isolated as described in Table 3, and the fatty acid composition was determined as described in the text. Values are mol % representing the mean ± standard deviation, n = 4 in each case.
[b]Not detectable.

TABLE 5

Phospholipids and Neutral Lipids in Spore Homogenates and Membranes[a]

	Phospholipids	Diacylglycerol[b]	Free fatty acid
Homogenate	80.9 ± 5.3	15.0 ± 4.4	4.2 ± 1.8
Membranes	83.2 ± 5.5	12.6 ± 2.8	4.3 ± 2.8

[a]Lipids were extracted from spore homogenates and membranes, separated by thin layer chromatography, transesterified and the fatty acids were measured by gas liquid chromatography as described in the text. Values are fatty acid mol % representing the mean ± standard deviation, n = 3 in each case.
[b]Diacylglycerol contained both 1,2-diacylglycerol and 1,3-diacylglycerol species.

contents of the neutral lipid fractions and phospholipids from both homogenates and membranes are shown in Table 5. The bulk of the fatty acids were in phospholipids as expected with a small amount of free fatty acids, but the amounts of diacylglycerols were surprisingly high. Such high levels of diacylglycerol could result from improper handling that allowed considerable phospholipid degradation.

We know spores contain a Ca^{++}-dependent phospholipase C activity (unpublished), which could produce diacylglycerol. However, we think that these neutral fractions may be more important than merely sample degradation for the following reasons. First, the amounts of diacylglycerols were similar in homogenates and purified membranes, but preparation times for those fractions are quite different: 20 min and 18 hr, respectively. Second, the addition of 1 mM EDTA during the entire isolation procedure did not alter the results. Third, in addition to 1,2-diacylglycerol, which could be the product of phospholipase C, 1,3-diacylglycerol was also present in almost equal amounts. The fatty compositions of both the 1,3-diacylglycerol and free fatty acids were significantly different from the 1,2-diacylglycerol and phospholipids (the latter two were similar to each other). Therefore, it is likely that 1,3-diacylglycerol and free fatty acids represent unique lipid fractions (unpublished). However, for the

purposes of this report, we have now accounted for all of the spore fatty acids; from the results in Tables 1 and 5 about 65% of the total spore fatty acids were in phospholipid, 15% in neutral lipids containing diacylglycerols and free fatty acids and the remaining 20% in other fractions in Table 1. In these other fractions, there were at least two classes of substances that contained fatty acids. One was a unique pool of phospholipids high in i-C14, i-C16 and i-C18 that was resistant to lipid solvent extraction (Nikolopoulou, M., and Vary, J.C., in preparation), which accounted for almost half of the fatty acids in the delipidated pellet. Another class was proteins with acylated fatty acids as described below.

Acylation of proteins with fatty acids. When delipidated spore membranes were separated by SDS-PAGE, phospholipids were found in the molecular weight positions between 6 and 15 kDa, but no phospholipids were found above 15 kDa (Nikolopoulou, M., and Vary, J.C., in preparation). However, the protein above 15 kDa did contain fatty acids that were found after eluting the proteins from the gel followed by dialysis, transesterification and GLC analysis. Most of the fatty acids (>50%) were n-C14, n-C16 and n-C18, but the amounts were too low to accurately quantitate. To determine what proteins contained fatty acids, spores were grown in [1-^{14}C]palmitate (10 μCi/ml). With spores grown in [^{14}C]palmitate, delipidated proteins were prepared and separated by SDS-PAGE followed by autoradiography (Fig. 1). Panel A, lane 1, shows several bands that were radioactive, suggesting that proteins might have palmitate attached, and lane 2 was the same except the proteins were boiled in sample buffer containing DTT before electrophoresis. Since the banding patterns were the same, the fatty acids were not attached by thioester bonds such as through a glycerol moiety. Shown in panel B, lanes 1, 2 and 3, are a control and the results of treating the delipidated membrane proteins with hyroxylamine by published methods (14). Almost all of the bands were gone, or at least significantly reduced, after NH_2OH at pH 10, suggesting that the palmitate was in ester linkage to suggesting that the palmitate was in ester linkage to proteins and very little in amide or ether linkage. None of the above treatments changed the Coomassie blue staining patterns of the gel. Direct transesterification of delipidated membrane proteins completely eliminated all radioactivity (which was soluble in $CHC1_3/CH_3OH$) and shows that the radioactively labeled bands were not a result of metabolism of palmitate to amino acids. These data are the first to show direct acylation of fatty acids to proteins in bacilli. As in eukaryotes, the function of these post-translational modifications is unknown, but the hydrophobic fatty acids could serve as membrane anchors or even regulate catalytic activity.

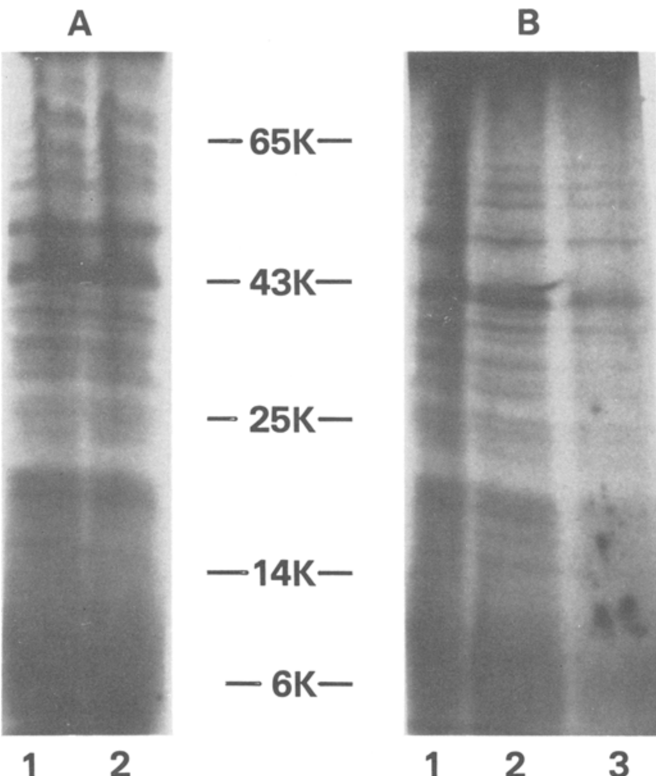

FIG. 1. Fatty acylated spore proteins. Spore were grown with [1-^{14}C]palmitate and disrupted, and the homogenate was delipidated by method B. The delipidated proteins (200 μg) were separated by SDS-PAGE followed by autoradiography. Panel A, prior to electrophoresis, samples were boiled without dithiothreitol (DTT) (lane 1) or with 1 mM DTT (lane 2); panel B, samples were treated with H_2O (lane 1), 1 M NH_2OH at pH 7 (lane 2) or 1 M NH_2OH at pH 10 (lane 3) as described (14), followed by electrophoresis and autoradiography as described in the text.

In summary, we have accounted for the fatty acids previously reported to be >50% in non-phosphatide fractions (3). By improved lipid extraction, 65% of the fatty acids were in phospholipids and 15% in neutral lipids. The remainder were in the aqueous phase (6%) and interphase (8%) of the lipid extraction and 6% was in the delipidated pellet. The phospholipids were quantitated and analyzed for fatty acid composition, and no phospholipid contained a unique composition. Finally, fatty acids were also found in the delipidated pellet that were acylated to proteins.

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Abnormal Metabolism of Polyunsaturated Fatty Acids and Phospholipids in Diabetic Glomeruli

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Studies were done on changes in phospholipid content and fatty acid composition of phospholipids and on the role of the acylation pathway in synthesis of phospholipids in the development of abnormal fatty acid composition in the glomeruli of rats 2 and 10 mo after induction of diabetes with streptozotocin. The proportions of individual phospholipids in the glomeruli of rats were not changed 2 mo after induction of diabetes, but the proportion of phosphatidylethanolamine (PE) decreased and that of sphingomyelin increased 10 mo after induction of diabetes. In contrast, in liver the proportion of PE was increased and that of phosphatidylcholine was decreased. These results showed that changes of individual phospholipids in glomeruli were time-dependent and tissue-specific. Two mo after induction of diabetes, the main change in the phospholipid fatty acid composition of diabetic glomeruli was a decrease in arachidonic acid (AA); the main change in serum free fatty acids (FFA) was an increase in linoleic acid (LA) and a decrease in AA. Ten mo after induction of diabetes, the main changes in the phospholipid fatty acid composition of glomeruli were an increase in LA and a decrease in AA; the main change of the serum FFA composition was a decrease in AA. Thus, the fatty acid composition of glomerular phospholipids was not directly correlated to that of the serum in diabetic rats. Acyl-CoA synthetase and acyltransferase activities increased in diabetic glomeruli with either AA or LA as substrate, but activity toward LA increased more at 2 mo after induction of diabetes. Acyl-CoA synthetase activity increased in diabetic glomeruli with LA as substrate, but that did not change with AA as substrate at 10 mo after induction of diabetes. Furthermore, acyltransferase activity decreased in diabetic glomeruli with AA as substrate, although that did not change with LA as substrate at 10 mo after induction of diabetes.
Lipids 22, 704–710 (1987).

The content of arachidonic acid (AA) in phospholipids of the serum and several tissues is reported to decrease, but linoleic acid (LA) increases in experimental diabetes mellitus (1-6). Holman et al. (3) reported that one cause of these changes was a decrease in insulin-dependent desaturase activity for polyunsaturated fatty acids (PUFA). However, factors in individual tissues may also be important in changes of these fatty acid levels, because heart, liver, skin, testis, aorta and kidney may differ from each other and from serum (3,4). These findings indicate that glomerular metabolism of PUFA is important for development of an abnormal fatty acid composition in experimental diabetes mellitus.

Serum PUFA such as AA are incorporated into tissue phospholipids mainly through Lands' pathway in the liver (7). This pathway consists of 2 steps: first, activation of free fatty acids (FFA) to acyl-CoAs by acyl-CoA synthetase and then incorporation of the acyl-CoAs into phospholipids by acyl-CoA:1-acylglycerophospholipid (lysoPL) acyltransferase. In this work, we investigated whether Lands' pathway contributes to changes in fatty acid contents of glomeruli in experimental diabetes mellitus.

The proportion of phosphatidylethanolamine (PE) is reported to be increased with a decrease in that of phosphatidylcholine (PC) in the liver of diabetic rats (8). These changes were attributed to decreased methylation of PE to PC (9). But Clark et al. (5) reported that the contents of PE and PC are not changed in renal cortex, renal plasma membranes or glomeruli of diabetic rats. These data on changes in the composition in the glomeruli and the liver were obtained in the early stage of diabetes mellitus, when diabetic nephropathy was still not clear (10,11) (3-6 wk after the streptozotocin injection). Thickening of the glomerular mesangial matrix was seen 6 mo after induction of diabetes mellitus (12). Thus, there may be two stages of metabolism. The second aim of this work was to compare metabolism in an advanced stage of diabetic nephropathy with that in the early stage. In this work, we compared the metabolism of fatty acids and phospholipids in rats 2 mo and 10 mo after induction of diabetes mellitus. The results indicated the importance of tissue metabolism and the time dependence in development of the abnormalities seen in diabetic glomeruli.

MATERIALS AND METHODS

Chemicals. LA and AA were obtained from Nu-Chek-Prep (Elysian, MN). 1-Acylglycerophosphorylethanolamine (lysoPE) was obtained from Serdary Research Laboratories (Ontario, Canada). Adenosine triphosphate (ATP) and coenzyme A (CoA-SH) were from Sigma Chemical Co. (St. Louis, MO). Glutathione and Triton X-100 were from Wako Pure Chemical Industries Ltd. (Tokyo). [1-^{14}C]LA (52.6 mCi/mmol) and [1-^{14}C]AA (52.0 mCi/mmol) were from New England Nuclear (Boston, MA).

Induction of diabetes. Male Wistar rats, 2 mo old, weighing about 250 g, were given an injection through a tail vein of streptozotocin (60 mg/kg) in saline and were maintained on standard chow (Oriental Cobo Co., Tokyo) for 2 or 10 mo. Control rats were given standard chow for the same period.

Preparation of glomeruli. Kidneys were quickly removed from anesthetized rats and washed with ice-cold saline, and glomeruli were prepared by the method of Wakashin et al. (13).

Preparation of enzyme solution. Glomeruli from two kidneys were homogenized with 2 ml of ice-cold 0.25 M sucrose-5 mM Tris-HCl buffer (pH 7.4) in a teflon-glass homogenizer (10 strokes). The homogenate was centrifuged

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Abbreviations: AA, arachidonic acid; LA, linoleic acid; PUFA, polyunsaturated fatty acids; FFA, free fatty acids; lysoPL, 1-acylglycerophospholipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; lysoPE, 1-acylglycerophosphorylethanolamine; ATP, adenosine triphosphate; CoA-SH, coenzyme A; TLC, thin layer chromatography; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin; PA, palmitic acid; OA, oleic acid; SA, stearic acid.

at 800 × g for 5 min, and the supernatant was used for enzyme assay.

Measurement of acyl-CoA synthetase activity. Acyl-CoA synthetase activity was assayed as described previously (14). The characterization of this enzyme in glomeruli has been reported elsewhere (15).

Measurement of lysoPE acyltransferase activity. LysoPE acyltransferase activity was measured by a slight modification of the method of Kramer and Deykin (16). The mixture for assay of lysoPE acyltransferase contained 20 mM ATP, 200 μM CoA-SH, 50 mM $MgCl_2$, 150 mM Tris-HCl buffer (pH 7.4), 250 μM lysoPE, 200 μM FFA, 0.1 μCi [1-^{14}C]FFA and 0.1-0.3 mg protein of glomerular enzyme solution in a final volume of 0.25 ml. The reaction mixture was incubated for 45 min at 37 C. The reaction was stopped by addition of 4 ml of Folch's solution (chloroform/methanol, 2:1, v/v). Lipid was extracted by the method of Folch et al. (17) and PE was separated by thin layer chromatography (TLC, Merck, Darmstadt, FRG) (18). Radioactivity of PE was counted, and acyltransferase activity was calculated from the specific activity. Enzyme activity increased linearly with the amount of enzyme protein up to 800 μg and with the incubation period for up to 90 min.

Fatty acid compositions of serum FFA and glomerular phospholipids. Serum FFA was separated by TLC (first with isopropylether/acetic acid, 96:4, v/v; second with petroleum ether/diethylether/acetic acid, 90:10:1, v/v/v) (18). Glomerular lipids were extracted by the method of Folch et al., and then phospholipid was separated by unicil column chromatography. In some experiments, phospholipids were isolated by TLC (chloroform/methanol/petroleum ether/acetic acid/boric acid, 80:60:40:20:3.6, v/v/v/v/w)(19). This method does not resolve phosphatidylinositol (PI) from phosphatidylserine (PS).

The extracts were then evaporated under N_2 gas. Methylation of serum FFA was achieved by incubating the extracts with 0.2 ml of boron trifluoride methanol complex for 2 min at 100 C, and the methylated fatty acids were extracted with 2 ml of hexane. Hydrolysis of phospholipids and methylation of the fatty acids of glomeruli were achieved by incubating the extracts with 1 ml of 0.6 N NaOH in 100% methanol for 30 min at room temperature. After incubation, the mixture was neutralized with 6 N HCl, and the methylated fatty acids were extracted with 2 ml of hexane.

The fatty acid composition of serum FFA and glomerular phospholipids was analyzed by gas chromatography in a GC 7-A model apparatus (Shimadzu Co., Kyoto) equipped with a 2 m × 2 mm id glass column packed with 15% DEGS and with a flame ionization detector. The column temperature was 190 C. Nitrogen was used as carrier gas, and the flow rate was 50 ml/min. Peaks were identified by comparison with standards (Nu-Chek-Prep). The peak area was calculated electronically with a computing integrator (Chromatopac CRIA; Shimadzu Co., Kyoto). The values reported in Tables 3, 4 and 5 are mean area %.

Lipid analysis of glomeruli and liver. Before lipids were extracted, cholesterol acetate was added as an internal standard. Then lipids were extracted by the method of Folch et al. and analyzed with TLC and a flame ionization detector (20,21) using an Iatroscan TH-10 TLC/flame ionization analyzer (Diatron, Tokyo). Lipids were separated with the solvent system of hexane/ether/formic acid (54:5:0.05, v/v/v) for neutral lipids and chloroform/methanol/water (60:2:2, v/v/v) for phospholipid subfractions.

Measurement of protein. Protein was measured by the method of Lowry et al. (22).

Statistics. The significance of differences in mean values was evaluated by Student's t-test.

RESULTS

Animal conditions (Table 1). The body weights of diabetic rats were markedly less than those of controls 2 and 10 mo after injection of streptozotocin. The kidney weights of diabetic rats were not significantly different from those of controls.

The serum glucose levels of diabetic rats were markedly increased 2 and 10 mo after injection of streptozotocin. However, the glucose levels were higher after 2 mo than after 10 mo. The total cholesterol levels of diabetic rats were significantly higher after 2 mo, but lower after 10 mo than those of controls. The mean triglyceride levels of

TABLE 1

Data on Control and Diabetic Rats

	2 Months[a]		10 Months[a]	
	Control (n = 5)	Diabetic (n = 5)	Control (n = 5)	Diabetic (n = 5)
Weight (g)				
Whole body	448 ± 24.1[b]	196 ± 14.7[c]	547 ± 29.9	172 ± 7.6[c]
Kidney	2.5 ± 0.1	2.6 ± 0.1	3.0 ± 0.1	2.4 ± 0.1
Serum (mg/dl)				
Glucose	110 ± 4.4	905 ± 42.4[c]	136 ± 5.3	583 ± 15.6[c]
Total cholesterol	121 ± 8.0	145 ± 4.9[d]	147 ± 5.8	117 ± 4.0[d]
Triglyceride	111 ± 7.1	222 ± 51.8	287 ± 29.9	226 ± 21.9
Phospholipid	180 ± 10.7	259 ± 8.0[d]	—	—

[a]Time after induction of diabetes.
[b]Values are means ± SEM.
[c]$p < 0.01$.
[d]$p < 0.05$.

TABLE 2

Lipid Contents of Glomeruli from Control and Diabetic Rats

	2 Months[a]		10 Months[a]	
	Control (n = 5)	Diabetic (n = 4)	Control (n = 5)	Diabetic (n = 5)
	μg/mg glomerular protein			
Triglyceride	5.2 ± 0.5[b]	8.0 ± 0.9[d]	3.0 ± 0.1	5.0 ± 0.8
Cholesterol ester	10.7 ± 0.6	9.9 ± 3.9	4.0 ± 0.4	4.0 ± 0.8
Free cholesterol	65.2 ± 10.3	56.5 ± 4.9	46.0 ± 2.6	48.0 ± 3.1
Phospholipids	322 ± 30.7	337 ± 35.0	234 ± 24.1	240 ± 17.4
PC	38.8 ± 1.1[e]	40.6 ± 1.5	42.7 ± 2.3	42.6 ± 2.3
PI + PS	8.7 ± 1.4	8.6 ± 1.5	8.4 ± 1.3	6.8 ± 1.3
PE	30.3 ± 1.7	26.5 ± 1.3	24.6 ± 1.6	12.8 ± 1.3[c]
Sph	23.2 ± 2.2	24.1 ± 1.9	25.1 ± 2.1	40.8 ± 2.2[c]

PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; Sph, sphingomyelin.
[a]Time after induction of diabetes.
[b]Values are means ± SEM.
[c]$p < 0.001$.
[d]$p < 0.05$.
[e]% of total phospholipid pool.

TABLE 3

Fatty Acid Composition of Serum Free Fatty Acids in Control and Diabetic Rats

	2 Months[a]		10 Months[a]	
Fatty acid	Control (n = 5)	Diabetic (n = 5)	Control(n = 5)	Diabetic (n = 4)
16:0	22.2 ± 1.2[b]	17.4 ± 1.2[e]	14.9 ± 0.2	17.1 ± 0.8[e]
18:0	8.0 ± 0.8	7.9 ± 0.9	8.2 ± 0.2	8.7 ± 0.4
16:1(n-7)	2.2 ± 0.3	nd	4.4 ± 0.3	4.8 ± 1.3
18:1(n-9)	14.9 ± 1.4	10.1 ± 0.6[e]	12.7 ± 0.7	12.9 ± 0.3
18:2(n-6)	24.8 ± 0.5	35.0 ± 2.2[d]	16.2 ± 1.4	19.1 ± 1.8
20:3(n-6)	0.2 ± 0.1	0.8 ± 0.2	0.2 ± 0.1	0.5 ± 0.1
20:4(n-6)	10.5 ± 0.3	7.5 ± 0.3[d]	27.2 ± 1.7	19.3 ± 1.5[e]
22:4(n-6)	0.7 ± 0.1	0.5 ± 0.3	1.2 ± 0.6	0.7 ± 0.6
20:5(n-3)	0.5 ± 0.1	0.4 ± 0.1	nd	nd
22:5(n-3)	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.1
22:6(n-3)	3.7 ± 0.1	5.5 ± 0.1[c]	3.1 ± 0.2	3.9 ± 0.3

nd, Not detectable.
[a]Time after induction of diabetes.
[b]Values are area %, means ± SEM.
[c]$p < 0.001$.
[d]$p < 0.01$.
[e]$p < 0.05$.

diabetic rats were also higher after 2 mo and lower after 10 mo than those of controls. The serum lipid levels of controls were slightly, but significantly, higher after 10 mo than after 2 mo.

Lipid contents of glomeruli from control and diabetic rats (Table 2). The major lipids of glomeruli were free cholesterol and phospholipids. There were no significant differences in their contents (μg/mg glomerular protein) in control and diabetic rats after either 2 or 10 mo. The relative amounts of PC and PI plus PS were also similar in normal and diabetic rats at both times. In diabetic rats after 10 mo, the relative amount of PE was significantly decreased and that of sphingomyelin (Sph) was significantly increased, although these changes were not clear after 2 mo. Serum lipids were not correlated with tissue lipids (Table 1). The contents of free cholesterol and phospholipids on the basis of glomerular protein were lower after 10 mo than after 2 mo.

Serum FFA in diabetic rats (Table 3). Serum FFA were analyzed to see whether their composition reflected that in the glomeruli. The main saturated FFA in the serum was palmitic acid (PA), the main monoene was oleic acid (OA) and the main PUFA were LA and AA (Table 3, control). Diabetic rat serum contained more LA but less AA than controls at either 2 or 10 mo, but the increase in LA and the decrease in AA were most profound at 2 mo and the decrease in AA was most prominent at 10 mo. A slight increase in docosahexaenoic acid was observed at both times.

Control animals showed age-dependent changes in serum

TABLE 4

Fatty Acid Composition of Glomerular Phospholipids from Control and Diabetic Rats

	2 Months[a]		10 Months[a]	
Fatty acid	Control (n = 5)	Diabetic (n = 4)	Control (n = 5)	Diabetic (n = 5)
16:0	16.0 ± 1.1[b]	16.2 ± 1.0	19.6 ± 0.3	19.3 ± 1.3
18:0	20.7 ± 0.4	19.0 ± 0.3	20.9 ± 0.8	22.1 ± 0.5
16:1(n-7)	0.8 ± 0.1	0.7 ± 0.2	1.6 ± 0.4	0.6 ± 0.1
18:1(n-9)	9.2 ± 0.8	9.0 ± 1.0	10.4 ± 0.2	10.0 ± 0.9
18:2(n-6)	10.6 ± 0.1	15.4 ± 0.8[d]	11.7 ± 0.4	19.5 ± 0.4[c]
20:3(n-6)	1.1 ± 0.2	1.9 ± 0.3	nd	nd
20:4(n-6)	25.9 ± 0.4	14.9 ± 1.2[c]	22.4 ± 0.4	13.9 ± 0.8[c]
20:5(n-3)	nd	nd	0.2 ± 0	0.3 ± 0.1
22:5(n-3)	1.1 ± 0.1	2.0 ± 0.5	1.6 ± 0.2	1.4 ± 0.1
22:6(n-3)	2.1 ± 0.5	1.8 ± 0.3	2.4 ± 0.1	2.5 ± 0.1

nd, Not detectable.
[a]Time after induction of diabetes.
[b]Values are area %, means ± SEM.
[c]$p < 0.001$.
[d]$p < 0.01$.

TABLE 5

Fatty Acid Composition of Individual Phospholipids of Glomeruli from Control and Diabetic Rats at 10 Months

	PC		PE		PI + PS	
Fatty acid	Control (n = 3)	Diabetic (n = 3)	Control (n = 3)	Diabetic (n = 3)	Control (n = 3)	Diabetic (n = 3)
16:0	36.3 ± 1.9[a]	28.6 ± 1.3[b]	9.7 ± 0.3	6.5 ± 1.0[b]	6.3 ± 1.5	7.8 ± 2.5
18:0	8.2 ± 1.7	10.2 ± 0.7	21.4 ± 0.7	17.3 ± 2.7	30.0 ± 0.4	30.6 ± 2.5
16:1(n-7)	1.5 ± 0.2	1.4 ± 0.3	0.7 ± 0.2	0.7 ± 0.1	1.3 ± 0.6	0.8 ± 0.1
18:1(n-9)	10.9 ± 0.2	10.2 ± 0.6	8.5 ± 0.4	11.1 ± 3.8	11.7 ± 2.4	10.3 ± 2.6
18:2(n-6)	12.4 ± 0.4	24.7 ± 3.1[b]	5.1 ± 0.4	12.3 ± 3.0	8.6 ± 1.0	10.7 ± 2.4
20:3(n-6)	0.3 ± 0.1	0.9 ± 0.1[b]	0.7 ± 0.3	0.7 ± 0.3	0.8 ± 0.1	2.0 ± 0.9
20:4(n-6)	13.0 ± 2.1	10.3 ± 0.9	37.2 ± 0.2	25.0 ± 3.0[b]	20.0 ± 1.1	21.4 ± 4.0
22:4(n-6)	0.4 ± 0.3	0.3 ± 0.1	3.5 ± 0.8	1.9 ± 0.1	2.4 ± 0.7	1.8 ± 0.1
22:5(n-3)	0.2 ± 0.1	0.5 ± 0.1[b]	0.9 ± 0.4	0.9 ± 0.5	0.9 ± 0.1	1.1 ± 0.2
22:6(n-3)	1.5 ± 0.5	2.2 ± 0.1	4.4 ± 0.1	3.9 ± 0.5	2.1 ± 0.1	2.4 ± 0.6

PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.
[a]Values are area %, means ± SEM.
[b]$p < 0.05$.

FFA. AA content increased almost three-fold between 2 and 10 mo, while LA and PA content decreased to a lesser degree.

Fatty acid composition of glomerular phospholipids from diabetic rats (*Table 4*). The main saturated fatty acids were PA and stearic acid (SA), the main monoene was OA and the main polyenes were LA and AA. The relative amounts of fatty acids in glomerular phospholipids were different from those in serum FFA. Diabetes lowered AA and increased LA in glomeruli at 2 or 10 mo. No age-dependent changes in free fatty acids in glomeruli were observed in controls.

Fatty acid composition of individual phospholipids of glomeruli from diabetic rats after 10 mo (*Table 5*). The fatty acid composition of individual phospholipids was analyzed to determine in which subfraction these changes occurred. LA was mainly present in PC and AA mainly in PE. In diabetes mellitus, LA was increased in PC, and the mean level of LA in PE was also increased slightly, but not significantly. AA decreased only in PE and did not change significantly in other fractions. PA was significantly decreased in PC and PE. There was no significant difference in the contents of PI + PS in control and diabetic glomeruli.

Acyl-CoA synthetase and lysoPE acyltransferase activities of glomeruli from diabetic rats. As changes of both LA and AA were observed in PE, the pathway for synthesis of PE from FFA was investigated. LA and AA were used as substrates because the contents of both in PE changed. In diabetic rats at 2 mo, the acyl-CoA synthetase

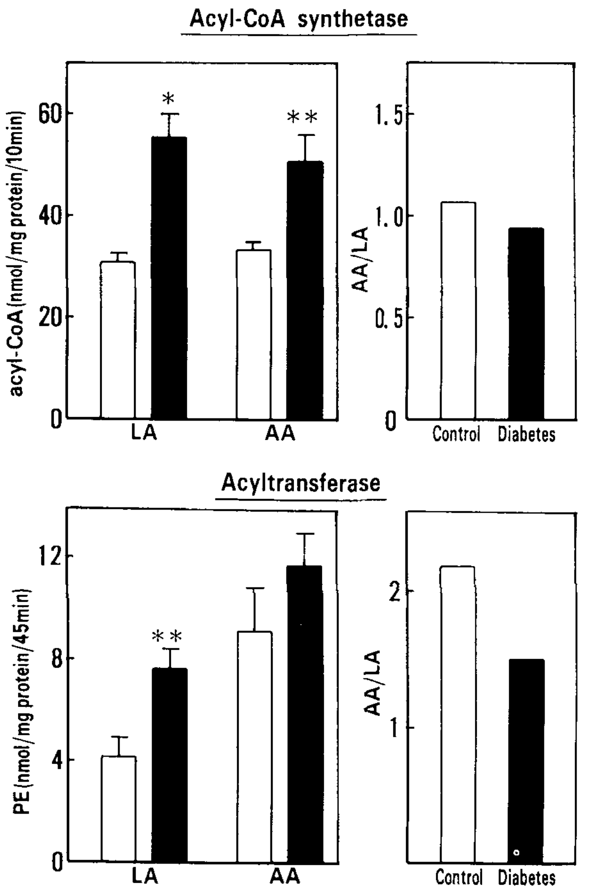

FIG. 1. Acyl-CoA synthetase and lysoPE acyltransferase activities of glomeruli from control and diabetic rats at 2 mo. Results are shown as means ± SEM for five animals. Asterisks above bars indicate significant differences from control values: *, $p < 0.01$; **, $p < 0.05$. LA, linoleic acid; AA, arachidonic acid; PE, phosphatidylethanolamine; □, control rats; ■, diabetic rats.

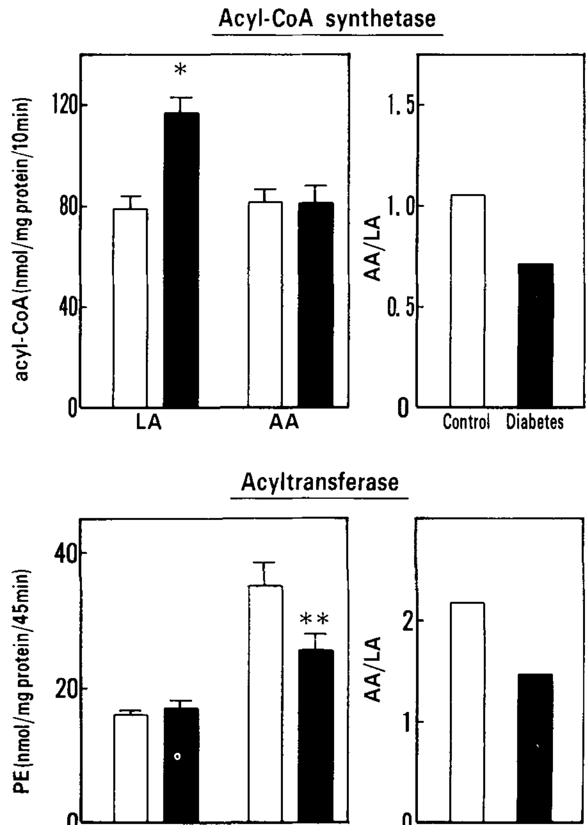

FIG. 2. Acyl-CoA synthetase and lysoPE acyltransferase activities of glomeruli from control rats and diabetic rats after 10 mo. Results are means ± SEM for five animals. The asterisks above bars indicate significant differences from control values: *, $p < 0.01$; **, $p < 0.05$; □, control rats; ■, diabetic rats.

activities with LA and AA as substrates were significantly higher than those in control rats, but the ratio of arachidonyl-CoA synthesis/linoleyl-CoA synthesis was not significantly different in control and diabetic rats (Fig. 1). In diabetic rats after 10 mo, the acyl-CoA synthetase activity with LA as substrate was significantly increased but that with AA as substrate was not changed, and so the ratio of arachidonyl-CoA synthesis/linoleyl-CoA synthesis was decreased (1.04 vs 0.70) (Fig. 2). In diabetic rats after 2 mo, lysoPE acyltransferase activity with LA as substrate was increased significantly, but that with AA as substrate was increased only slightly; consequently the ratio of PE synthesis from AA/PE synthesis from LA was decreased (2.17 vs 1.51) (Fig. 1). In diabetic rats after 10 mo, lysoPE acyltransferase activity with LA as substrate was not changed but that with AA as substrate was decreased significantly, and so the ratio of PE synthesis from AA/PE synthesis from LA was decreased (2.17 vs 1.47) (Fig. 2).

Phospholipid content of the liver of diabetic rats after 10 months (Fig. 3). Liver phospholipids 10 months after induction of diabetes were examined to determine whether changes in glomeruli are tissue-specific. The proportion of PE was increased with reciprocal decrease in the proportion of PC in the liver of diabetic rats after 10 mo.

DISCUSSION

We found that the proportions of individual phospholipids in the glomeruli of diabetic rats were not changed after 2 mo, in agreement with Clark et al. (5), but that the proportion of PE was decreased and that of Sph was increased reciprocally after 10 mo (Table 2). Therefore, the changes of individual phospholipids in the glomeruli of diabetic rats were time-dependent. Fass and Carter (8) reported that PE increases in rat liver 3 wk after induction of diabetes. We observed an increase in PE and a decrease in PC in livers of 10-mo diabetics. These results indicate that changes of individual phospholipids in glomeruli were tissue-specific. The enzyme methylating PE to form PC is thought to be present in the liver and to show reduced activity in diabetes mellitus (9). It is unknown whether this enzyme exists in glomeruli and whether the methylation pathway is important for synthesis of PC in glomeruli. Our results suggest that this pathway is not so important for the synthesis of PC in the glomeruli as in the liver. The reason that the proportion of PE decreased and that of Sph increased in the glomeruli in the later stage of diabetes is not clear. The

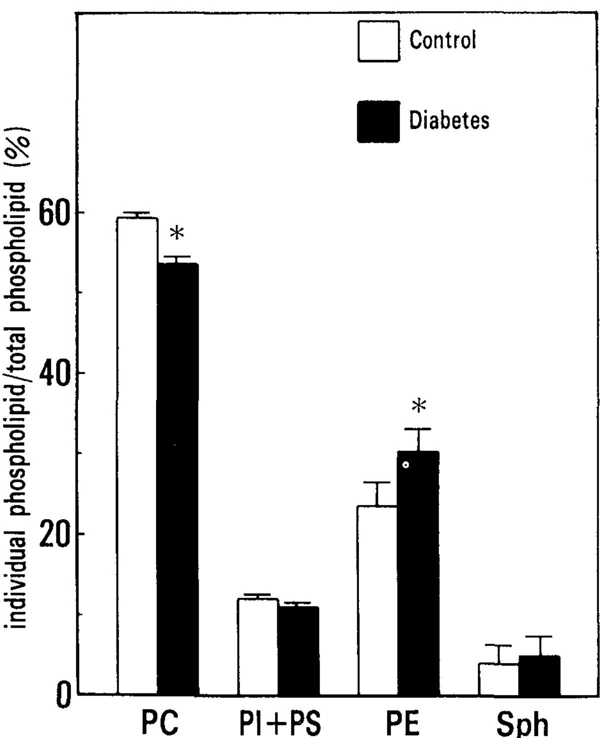

FIG. 3. Phospholipid content of the liver of diabetic rats after 10 mo. Results are means ± SEM for five rats. The asterisks above the bar indicate significant differences from the control value: *, $p < 0.05$. PC, phosphatidylcholine; PI + PS, phosphatidylinositol + phosphatidylserine; PE, phosphatidylethanolamine; Sph, sphingomyelin. □, control rats; ■, diabetic rats.

diabetic state might (i) inhibit de novo PE synthesis and (ii) either stimulate Sph synthesis or inhibit sphingomyelinase, or both. However, the precise effects of diabetes mellitus on these pathways are not known. Another possibility is that Sph-rich and PE-poor cell populations increased in the glomeruli of diabetic rats. Glomeruli prepared by the method used in this work could contain endothelial, mesangial and epithelial cells. Mesangial cells may proliferate in the diabetic state (23).

The fatty acid composition of phospholipids depends on the balance of their synthesis and degradation. The deacylation-reacylation cycle is especially important for PUFA (7). In this work, we examined the synthetic pathway (acylation). Our results showed that in diabetes mellitus there was an imbalance of utilization of PUFA in the acyl-CoA synthetase-lysoPE acyltransferase system: incorporation of LA into PE was preferential to that of AA in diabetic glomeruli both at 2 and 10 mo (Figs. 1 and 2). Our results indicate that this abnormal metabolism could contribute to the abnormal fatty acid composition of phospholipids in both early and late stages of diabetes mellitus. An AA-specific acyl-CoA synthetase was reported to be present in platelets (24), and its presence explains the high and selective incorporation of AA into phospholipids in platelets in spite of the low serum AA concentration. However, we could not detect an AA-specific acyl-CoA synthetase in glomeruli (15), and Clark et al. reported that acyl-CoA synthetase activities have little difference between LA and AA as substrates in normal or diabetic renal homogenates (25). On the contrary, the Michaelis coefficient (Km) of acyl-CoA synthetase for LA was lower and maximum velocity (Vmax) was higher than those for AA (Km for LA, 12.8 μM; Km for AA, 19.3 μM; Vmax for LA, 152 nmol/mg protein/10 min; Vmax for AA, 115 nmol/mg protein/10 min) (15). Thus, LA is a better substrate than AA for glomerular acyl-CoA synthetase. This and the imbalance in the acyl-CoA synthetase-lysoPE:acyltransferase system in the diabetic state indicate that glomeruli cannot counteract the abnormal FFA composition in diabetic serum at the level of uptake of FFA into the tissue and in fact may make it worse.

The mechanism of the imbalance in PE synthesis is not clear. In preliminary experiments, we found that the PE and Sph contents affect the enzyme activities in different ways with different substrate fatty acids. Possibly microenvironments of the enzyme and substrates may be important; we are now examining this possibility.

The changes observed in the phospholipids of diabetic glomeruli could affect either membrane functions or eicosanoid metabolism. The decrease in the proportion of PE, the increase in that of Sph and the decrease in double bonds of fatty acids in phospholipids could all result in decreased fluidity of glomerular cell membranes (5,26,27). Mead (28) reported that membrane proteins are easily released from membranes in these conditions, with consequent decrease in integrity of the membranes.

AA released from phospholipids was reported to be metabolized through the cyclooxygenase and lipooxygenase pathways in glomeruli (29-31). The syntheses of prostaglandins are affected not only by enzymes such as phospholipase and cyclooxygenase, but also by the AA content of phospholipids (32). Although several roles of prostaglandins in glomeruli, such as inhibition of platelet aggregation in glomerulonephritis (33) and regulation of angiotensin metabolism (34), have been proposed, little is known about the roles of these metabolites in formation of diabetic nephropathy. Rogers and Larkins (35) reported that the productions of PGI_2 and PGE_2 from endogenous AA were not decreased in the glomeruli of diabetic rats after 5 wk, in spite of a decrease in AA in phospholipids (5). It is not known whether PG synthesis decreases in the late stage. The decrease in AA in diabetic glomeruli was mainly in that of PE at 10 mo (Table 5). The origin of AA is not known; if PC is the main source of AA, no changes of PG may occur, but if PE is the main source of AA, then PG production may decrease. The glomeruli used in this work could contain various cell populations, such as endothelial, mesangial and epithelial cells. If AA in phospholipids and the release of AA from phospholipids were decreased in endothelial cells, the production of PGI_2 should decrease, thus facilitating thrombus formation (36). Further investigations are needed to correlate our data with results on diabetic nephropathy.

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Enteral Absorption in Man of Eicosapentaenoic Acid in Different Chemical Forms

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After administering the equivalent of 1 g of eicosapentaenoic acid (EPA) in four different chemical forms, the kinetics of EPA incorporation into plasma triglycerides (TG) were compared by gas liquid chromatography on a capillary column following separation of the lipid fraction by thin layer chromatography.

EPA incorporation into plasma TG was markedly smaller and later when EPA was administered as an ethyl ester rather than as EPA free fatty acid, EPA arginine salt or 1,3-dioctanoyl-2-eicosapentaenoyl glycerol (2-EPA). Our results and the data in the literature are compatible with the hypothesis that 2-EPA is absorbed with minimum hydrolysis and escapes random distribution between the other positions of the glycerol molecule during the absorption process.

Lipids 22, 711–714 (1987).

Dietary fat modification is now considered to be an effective tool for modifying the phospholipid fatty acid composition of cell membranes, especially in blood platelets. The effect of dietary manipulation and the metabolism of polyunsaturated fatty acids (PUFA) have been extensively studied in the aim of influencing the physiological responses mediated by the oxygenated metabolites of the eicosapolyenoic acid cascade.

Several studies have been devoted to the action and metabolism of eicosapentaenoic acid (EPA) in animals or in man, especially its antithrombotic effect (1-7). Owing to the greater stability of esterified PUFA vs free fatty acids, and to the potential toxicity of methanol released by methyl ester hydrolysis, many of the studies concerning dietary manipulation with EPA have used the ethyl ester of this acid, as in a recent study by Tamura et al. (8). However, some data in the literature raise questions about the relative difficulty of its hydrolysis by pancreatic lipase (9-11); this difficulty could result in some impairment of its subsequent intestinal absorption.

For this reason, we compared in this study the kinetics of EPA incorporation into plasma triglycerides (TG) in man after ingestion of four chemical forms of EPA: ethyl ester, free fatty acid, arginine salt and triglyceride (1,3-dioctanoyl-2-eicosapentaenoyl glycerol [2-EPA]). The kinetics of this incorporation after EPA ethyl ester ingestion differed strikingly from the kinetics following ingestion of the three other forms.

MATERIALS AND METHODS

Reagents. Free fatty acid, arginine salt, ethyl ester of EPA and 2-EPA of pure analytical grade were generously provided by Roussel-Uclaf Laboratories (Romainville, France).

The purity of the chemical forms was checked by gas chromatography-mass spectrometry; when useful, this was completed by NMR spectroscopy.

Organic solvents, hexane (Uvasol), chloroform, methanol, inorganic compounds and 2′,7′-dichlorofluorescein were from E. Merck (Darmstadt, FRG). The lipid and fatty acid standards were from Fluka (Buchs, Switzerland) or Sigma Chemical Co. (St. Louis, MO).

Experimental protocol. Eight normal volunteers, 25-29 years of age, were given the equivalent of 1 g of EPA per os at 8 a.m. after an overnight fast; immediately afterward they ate a light lipid-free breakfast. Under the same conditions, four of the subjects received the four chemical forms of EPA, each form taken at subsequent 1-wk intervals. The other four received only two forms, the free fatty acid and 2-EPA.

Blood samples (7 ml on ethylene diamine tetraacetate) were taken 0, 1, 2, 3, 4, 5, 6, 9, 12 and 24 hr after EPA ingestion. At 6 and 12 hr the samples were taken just before meals of low lipid content, and at 0 and 24 hr after an overnight fast.

Analytical methods. Plasma lipids were extracted by chloroform/methanol according to the method of Folch et al. (12). The lipid classes were separated by thin layer chromatography on Kieselgel 60 F 254 (Merck) using hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as the developer. The lipid fractions were detected under UV light after spraying the plates with 2′,7′-dichlorofluorescein. The TG fraction was scraped from the plates and transesterification was performed for 30 min at 80 C in methanol/sulfuric acid (19:1, v/v) without extraction from the gel. The methyl esters extracted with hexane were analyzed by gas liquid chromatography using a Fractovap 2900 (Erba Science) chromatograph, FFAP capillary columns (25 m × 0.32 mm) and a flame ionization detector at 250 C. The injection temperature was 230 C, and the oven

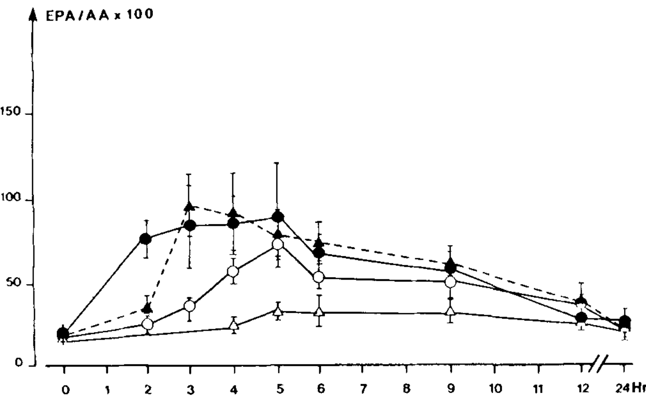

Fig. 1. EPA/AA × 100 ratio variation in plasma TG; mean value (± SEM) after ingestion of 1 g of EPA in the form of 2-EPA (○) free EPA (▲), arginine EP (•) and ethyl EP (△).

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Abbreviations: PUFA, polyunsaturated fatty acids; AA, arachidonic acid; EPA, eicosapentaenoic acid; 2-EPA, 1,3-dioctanoyl-2-eicosapentaenoyl glycerol; EP, eicosapentaenoate; TG, triglyceride.

was programmed to increase the temperature 10 C/min from 50 C to 150 C, and then 2 C/min from 150 C to 200 C. The esters were identified by their retention times with respect to pure fatty acid standards. The relative proportions of EPA and arachidonic acid (AA) were evaluated using a digital integrator (I.C.A.P-5 or Spectra-physics).

RESULTS

After 1 g EPA was ingested, its absorption was followed by substantial incorporation into plasma TG characterized by a rise in the ratio of EPA to AA in the plasma lipid fraction. The different absorption rates of EPA as given in the four different chemical forms were estimated from the corresponding variations of the ratio of EPA to AA in plasma TG.

Figure 1 compares the variation of the mean values of the ratio of EPA to AA in plasma TG after ingestion of the four different chemical forms. There is a striking difference among the kinetics observed after administration of EPA as ethyl ester and the three other chemical forms. When administered as ethyl ester, the rise of EPA in plasma TG was about three times less and occurred at least 3 hr later than with the three other chemical forms. The difference between EPA ethyl ester and the other forms is statistically significant ($p < 0.05$ at 5 and 6 hr), as is the increase in EPA/AA after ethyl ester ingestion ($p < 0.05$ at 5, 6 and 9 hr. vs control at 0 hr).

Tables 1-4 detail the individual variations of the ratio of EPA to AA in plasma TG after ingestion of the four different chemical forms of EPA.

After ingestion of an amount of the TG 2-EPA equivalent to 1 g of EPA, the ratio of EPA to AA increased in all eight subjects (Table 1). The maximum (3 to 10 times the basal value) was observed 4 or 5 hr after ingestion of TG. The maximum of the mean values (four to five times the mean basal value) was observed between 4 and 5 hr.

When the subjects were given 1 g of free EPA (Table 2), the increase in the ratio of EPA to AA was rapid; the maximum occurred at 3 hr and reached 5 to 12 times the initial value (mean value 5.5).

The variation of the ratio of EPA to AA after ingestion of an equivalent of 1 g of EPA as arginine salt was similar to that of 2-EPA; the maximum of the mean value is five times the basal value and occurred at 5 hr.

After ingestion of the ethyl ester of EPA, the ratio of EPA to AA in plasma TG rose slowly, and the maximum of the mean value was only two times higher than the basal value and was delayed until 9 hr.

TABLE 1

EPA/AA × 100 Ratio Variation in Plasma TG After Ingestion of 1 g of EPA in the Form of 2-EPA ($\overline{X}$ ± SEM)

Time (hr)	Subjects								Mean ± SEM		
	1	2	3	4	5	6	7	8	$\overline{X}$		
0	15	22	20	15	15	10	10	12	15	±	1,5
2			37		15	15	12	25	21	±	4,6
3				42	50	15	10	25	28,5	±	7,7
4	50	48	150	27	65	50	25	25	55	±	14,5
5	100	140	72	60	50	65	30	30	68	±	13
6	60	85	56	32	40	70	30	35	51	±	7
9		90	55	45	50	40	25	15	46	±	9
12	45	70	60	30	20	20	20	15	35	±	7,4
24	30	22	15	35	10	25	15	12	21	±	3

TABLE 2

EPA/AA × 100 Ratio Variation in Plasma TG After Ingestion of 1 g of free EPA ($\overline{X}$ ± SEM)

Time (hr)	Subjects								Mean ± SEM		
	1	2	3	4	5	6	7	8	$\overline{X}$		
0	22	22	20	22	20	22	12	9	17,4	±	2
2			20	41	45	25	17		30	±	5,6
3		172	140	131	100	65	25	30	95	±	21
4	52	250	62	68	65	145	35	37	89	±	26
5	57	170	56	77	65	95	46	46	76	±	15
6	107	110	36	47	60	85	55	70	71	±	9,6
9	83	75	46	50	45	95	50	20	58	±	8,6
12	55	62	46	50	20	30	20	15	37	±	6
24	32	52	22	15	10	25	15	12	22	±	3

DISCUSSION

The percentage of AA in plasma TG can be considered constant over the 24-hr period studied here, since this fatty acid was not supplied by food during this period. Under these conditions, the kinetics of EPA absorption can be estimated from the variation of the ratio of EPA to AA in plasma TG. This method has been used successfully by others (13). Our results show significant modifications of the ratio of EPA to AA in plasma TG after ingestion of the equivalent of 1 g of EPA in different chemical forms.

In spite of several individual variations, common characteristics clearly appeared, e.g., there was a 5- to 12-fold rise in the ratio of EPA to AA in 19 of the 20 experiments in which EPA was ingested as TG (2-EPA), free fatty acid and arginine salt. However, after ingestion of EPA ethyl ester, the ratio of EPA to AA increased only 1.5- to 3-fold over the basal value. This indicates that EPA was less incorporated into plasma TG after ingestion of the ethyl ester than after ingestion of the three other forms, but it does not imply that EPA ethyl ester is an ineffective therapeutic agent. The rise in the EPA/AA ratio is statistically significant at 5, 6 and 9 hr vs control at 0 hr. EPA ethyl ester has, in fact, been shown to be able to modify platelet function. The results obtained here with EPA ethyl ester are in agreement with data in the literature (14), e.g., a twofold rise above the basal level was obtained by Nagakawa et al. (5) even after repeated administration and by Tamura et al. (14) even after administration of a higher dose (3.5 g/day).

EPA was less readily incorporated into plasma TG after ingestion of ethyl ester than after ingestion of the TG (2 EPA), free fatty acid or arginine salt. The difference could result from impairments at the EPA ethyl ester hydrolysis step, at the EPA absorption step or from interference with some unknown process involved in the PUFA absorption mechanism. EPA is well absorbed when given as free fatty acid or arginine salt. The difference is possibly the result of poor hydrolysis of EPA ethyl ester by pancreatic lipase and a subsequent decrease in the incorporation of EPA ethyl ester into the mixed micelle. This resistance could result from both the EPA and the ethyl components of the molecule. An unusual resistance of the ethyl esters was in fact reported in 1958, i.e., the hydrolysis of ethyl oleate was found to be less than the hydrolysis of triolein (9), and the hydrolysis of ethyl esters was generally less than the hydrolysis of the homologous methyl esters (10,11). EPA structure is also involved in the relatively low susceptibility of EPA esters to hydrolysis by pancreatic lipase. Hydrolysis of eicosapentaenoyl or docosahexaenoyl glycerides is less than hydrolysis of oleyl glycerides (15,16) even when these fatty acids are situated at positions 1 and 3 on the triglyceride molecule, which are known to be the preferential sites of pancreatic lipase action (17). This resistance has been related to the proximity of the first double bond to the carboxyl group (17,18). Although the EPA glyceryl or docosahexaenoyl glycerol esters are resistant to hydrolysis by *pancreatic* lipase even at positions 1 and 3, the n-3 docosapentaenoyl glycerol is not (19). In the last compound, five methylene groups separate the ester function from the nearest double bond vs only two or three in the other molecules. Thus, $\Delta 4$ and $\Delta 5$ polyunsaturated fatty acids such as docosahexaenoic, eicosapentaenoic (and probably arachidonic) acids are scarcely released from their glyceride combinations by pancreatic lipase. On the other hand, $\Delta 7$ fatty acids such as n-3 docosapentaenoic acid are readily released by this enzyme and more easily available for further metabolism. A steric hindrance by the ω methyl group has also been suggested (16,19) to be the cause for the resistance of esters of the other fatty acids.

If such a resistance to the digestive enzymatic process impairs EPA ethyl ester hydrolysis, the same must hold for 2-EPA, especially when the ester bond is at position 2, which is resistant to pancreatic lipase. However, in all of the subjects studied, EPA incorporation into plasma TG was much greater after ingestion of 2-EPA than of ethyl ester. It is possible that the absorption of the ethyl ester takes place without prior enzymatic hydrolysis. This hypothesis is now under investigation.

TABLE 3

EPA/AA × 100 Ratio Variation in Plasma TG After Ingestion of 1 g of EPA in the Form of Arginine EP ($\overline{X}$ ± SEM)

Time (hr)	Subjects				Mean ± SEM		
	1	2	3	4	$\overline{X}$		
0	22	17	13	15	17	±	2
2			60	82	71	±	11
3		70	40	132	81	±	27
4	60	130	70	76	82,5	±	16
5	160	90	35	70	89	±	26
6	85	32	60	75	63	±	11
9	75	32	45	70	56	±	10
12	40	20		20	27	±	6,7
24	21	32	20	23	24	±	2,7

TABLE 4

EPA/AA × 100 Ratio Variation in Plasma TG After Ingestion of 1 g of EPA in the Form of Ethyl Ester ($\overline{X}$ ± SEM)

Time (hr)	Subjects				Mean ± SEM		
	1	2	3	4	$\overline{X}$		
0	16	16	12	12	14	±	1,2
2	—	—	—	—		—	
3	—	—	—	—		—	
4	22	21	15	22	20	±	2
5	35	32	14	36	28	±	4,7
6	35	30	20	26	28	±	3,1
9	38	32	17	38	31	±	5
12	30	25		20	25	±	2,9
24	30	20	18	20	22	±	2,7

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Sterol and Phospholipid Acyl Chain Alterations in *Saccharomyces cerevisiae* Secretion Mutants as a Function of Temperature Stress

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Analyses of free sterol, steryl ester and fatty acid components from yeast secretion mutants indicated that free and esterified sterol remained relatively constant over a growth range of 24 C to 34 C. The saturated fatty acid components (16:0 and 18:0) increased while the unsaturated fatty acids (16:1 and 18:1) decreased as the growth temperature increased. In secretory mutants, fatty acid composition changes are more pronounced than in the wild-type strain. A shift toward increased saturated and decreased unsaturated fatty acid was observed when cells were subjected to a 2-hr temperature upshift to 37 C. Steady-state fluorescence anisotropy data indicated that modifications to the lipid component of yeast plasma membrane produced lipid thermotropic transitions that were 3 C to 6 C higher in yeast cells subjected to thermal stress.
Lipids 22, 715–720 (1987).

The yeast *Saccharomyces cerevisiae* synthesizes and incorporates ergosterol and a mixture of phospholipids (sphingolipids, cardiolipin [CL], phosphatidylserine [PS], phosphatidylethanolamine [PE], phosphatidylcholine [PC] and phosphatidylinositol [PI] into membranes (1). The membrane lipid composition in this yeast has been shown to be dependent upon temperature, carbon source and lipid precursor supplement (1). By using a yeast sterol auxotroph, we have demonstrated coordinated regulation of fatty acid and phospholipid composition of the yeast plasma membrane (2) as a function of exogenous sterol. Maintenance of membrane integrity and stability under adverse environmental stress has been attributed to the presence of sterols in the plasma membrane (1,3). The stability of membranes during interactions with other membranes is important to the understanding of the mechanisms of membrane adhesion, fusion, cell division and detachment (4).

A body of evidence suggests a common eukaryotic protein secretory pathway that is mediated by membrane-bound structures (5,6). The isolation of temperature-sensitive secretory (sec) yeast mutants has shown that synthesis and secretion of extracellular and periplasmic protein are tightly coupled and appears to follow the typical eukaryotic secretory pathway (7–9). Yeast secretion mutants are conditionally restricted in expansion of plasma membrane. Restriction of plasma membrane expansion cannot be attributed to a cessation of phospholipid synthesis or altered phospholipid headgroup composition (10). Since the principal functional role for sterols in the plasma membrane appears to be structural, it seemed appropriate to determine whether any differences in sterol metabolism as a function of temperature stress mediated the defect in membrane formation.

MATERIALS AND METHODS

Strains and medium. The strains used in this study (Table 1) were derived from the wild-type strains X2180-1A and X2180-1B (7–9,11). The cells were grown on medium containing 0.67% yeast nitrogen base and 2% dextrose (YNBD); growth was followed turbidimetrically using a Klett-Summerson photoelectric colorimeter equipped with a green filter. Lipid extracts from wild-type and mutant yeasts cultured without shifting to the restrictive temperature (37 C) were compared to extracts from cells that had been incubated for 2 hr at the restrictive temperature. Based on differences in the sterol patterns found in these strains, a representative set of strains displaying the appropriate organelle phenotype (vesicle, Golgi, endoplasmic reticulum and no organelle accumulation) (8) upon temperature upshift was chosen for further analysis.

TABLE 1

Strain List

Strains	Genotype and organelle accumulated[a]
X2180-1A	a, *CUP1, SUC2, mal, gal2,* secretion wild-type, temperature-sensitive wild-type and parental for HMSF strains
X2180-1B	*α, CUP1, SUC2, mal, gal2,* secretion wild-type, temperature-sensitive wild-type and parental for HMSF strains
HMSF1[b]	*sec1-1* vesicles[c]
HMSF134	*sec5-24* vesicles
HMSF136	*sec6-4* vesicles
HMSF143	*sec9-4* vesicles and Berkeley bodies[d]
HMSF154	*sec11-7* none
HMSF169	*sec14-3* Berkeley bodies and vesicles (10), Golgi (11)
HMSF171	*sec15-1* vesicles
HMSF175	*sec17-1* endoplasmic reticulum and small vesicles[e]
HMSF178	*sec19-1* endoplasmic reticulum, Berkeley bodies, vesicles and small vesicles
HMSF179	*sec20-1* endoplasmic reticulum
HMSF180	*sec21-1* endoplasmic reticulum
HMSF183	*sec22-3* endoplasmic reticulum and small vesicles
HMSF190	*sec23-1* endoplasmic reticulum

[a]Intracellular membrane bound organelle (6–9,11).
[b]Genotype of all HMSF strains except for secretory mutation is a, *CUP1, SUC2, mal, gal2* (7,8,11).
[c]Vesicles 80–100 nm diameter.
[d]Berkeley bodies—membrane-enclosed toroids or cup-shaped structures and may be an altered Golgi body (19).
[e]Vesicles 40–60 nm diameter.

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Abbreviations: CL, cardiolipin; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; YNBD, medium containing 0.67% nitrogen base and 2% dextrose; DMSO, dimethylsulfoxide; TLC, thin layer chromatography; GLC, gas liquid chromatography; DPH, 1,6-diphenyl-1,3,5-hexatriene; FS, free sterol; SE, steryl ester.

Growth conditions. In temperature upshift experiments, cultures of X2180-1A, HMSF134, HMSF154, HMSF169 and HMSF190 were grown at 24 C to mid-log (100 Klett units) and the cultures divided in half. One-half of each culture was reincubated at 24 C and the other half at 37 C for 2 hr. For the 37 C-incubated cultures, a New Brunswick gyrotory water-bath shaker (Model G76) (water bath preset to 37 C) was used. Thermal equilibrium between the yeast culture medium and the water bath was reached within 15 min of placing the cultures in this water bath. At the end of 2 hr, yeast cells from the 24 C and 37–39 C cultures and from stationary phase cells cultured at 24 C were harvested and quantified by dry weight for lipid analyses and by wet weight for plasma membrane preparations. Temperature profile experiments from 26–40 C were carried out using a gradient incubator (Scientific Industries). The cells were cultured to stationary phase on YNBD over a temperature range of 26–40 C, and duplicate 10-ml samples were harvested for lipid analyses.

Lipid analyses. For lipid analysis, the cells were lyophilized and then either steamed in the presence of dimethylsulfoxide (DMSO) or subjected to alkaline saponification (12). In all cases, total cellular lipids were extracted using the procedure of Parks et al. (12). The thin layer chromatography (TLC) solvent system of Skipski and Barclay (13) was used to separate the extracted lipid components. The free sterols and sterol from steryl esters (saponified with 6% KOH in methanol, 2 hr, 80 C, and hexane extracted) were quantitated by gas liquid chromatography (GLC) [Applied Science Labs (Deerfield, IL), 3% SE-30, 235 C, N_2 carrier gas flow rate of 20 ml min^{-1}] (12). Phospholipids were extracted from TLC plates with chloroform/methanol (1:1, v/v) and rechromatographed using a two-dimensional TLC system (14). The fatty acid components of each phospholipid species were converted to their methyl ester equivalents (15,16) and then separated by GLC (Supelco 10% DEGS, 175 C, N_2 carrier gas flow rate of 20 ml min^{-1}).

Fluorescence anisotropy. For steady state fluorescence anisotropy studies, plasma membranes from yeast cultures were prepared using the *Oerskovia xanthineolytica* lyticase enzyme as described by Bottema et al. (17). Steady-state fluorescence anisotropy changes were measured with an SLM instrument series 8000 spectrofluorimeter using the lipophilic probe 1,6-diphenyl-1,3,5-hexatriene (DPH) (18). The steady-state polarization was calculated from the equation $P = (R - 1)/(R + 1)$, and by substitution of $R = I_{\parallel}/I_{\perp}$ into $r_s = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$ the steady-state fluorescence anisotropy was determined. Here, $I_{\parallel}$ and $I_{\perp}$ are the intensities of parallel and perpendicular polarized light, respectively. At each temperature point, triplicate measurements of the total photon counts were taken (>2 × 10^5 photons counted per data point measurement for statistical relevance). Temperature was determined within ±0.5 C and absorbance (460 nm) was less than 0.40 for all samples that contained DPH at a concentration of 1 μM in 10 mM Tris, 1 mM EDTA buffer, at pH 6.8. A linear least squares fit was used in determining the fluorescence anisotropy plot discontinuities.

Materials. DPH was purchased from Sigma Chemical Co. (St. Louis, MO). Solvents were from Fisher Scientific (Springfield, NJ) and were redistilled prior to use. Culture media and supplies were from Difco (Detroit, MI). A Varian 2700, a Hewlett-Packard 5890A gas-liquid chromatograph and a Beckman model 332 gradient liquid chromatograph were coupled to an IBM CS-9000 laboratory instruments computer and used for all GLC and high performance liquid chromatography (HPLC) analyses.

RESULTS

Screening of secretion mutants for altered sterol metabolism. Growth experiments (Fig. 1) showed that temperature sensitivity was detectable in the mutants after 2 hr of incubation at 37 C. The strains were inoculated into fresh medium and allowed to grow at 24 C until they reached 100 Klett units. At that time, each culture was shifted to 37 C for 2 hr, and the temperature was returned to 24 C. All of the mutants showed growth rate reductions following temperature upshift. By conducting the temperature downshift (37 C to 24 C), we were able to determine that these cells did not recover from thermal stress by returning to the original log phase growth rate in the same batch culture.

In one strain (HMSF134), it was noted that intracellular free sterol (FS) and steryl ester (SE) levels were comparable to that found in the wild-type (X2180-1A) when this strain was grown to stationary phase at 24 C. Intracellular SE (detected by GLC sterol analysis of the saponified SE fraction) did not accumulate under conditions of a temperature upshift as did the other representative strains, as evidenced by the high FS to SE ratio (FS/SE) (Table 2). Other secretion mutants, like the wild-type, began to accumulate SE after temperature upshift to 37 C. There were no large interstrain differences in total sterol between wild-type and the secretion mutant strains tested when cultured under the same temperature regimen (Table 3). For cells grown to stationary phase, the range of total sterol was 0.4 to 0.6 μg 10^8 $cells^{-1}$ (Table 3). A two- to fivefold increase in total sterol content was found in strains X2180-1A, HMSF154, HMSF169 and HMSF190 cultured to midexponential phase without temperature upshift compared to total sterol values for conditions of temperature upshift (37 C)

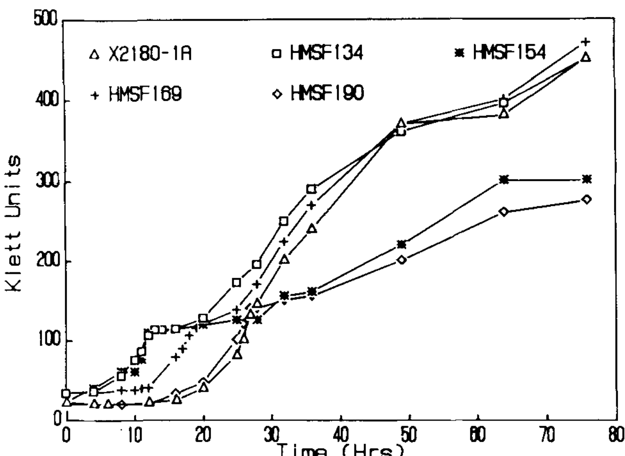

FIG. 1. Dual temperature shift growth of secretion mutants. Cells were cultured on yeast nitrogen base with dextrose to 100 KLett units at 24 C, then shifted to 37 C for 2 hr. The time period of the temperature upshift for each strain starts at 100 Klett units.

(Table 3). In cells subjected to temperature upshift, the total sterol content ranged from 0.4 to 0.9 μg 10^8 cells^{-1}, an amount not significantly different from cells grown to stationary phase (Table 3).

When sterols were compared by cell number (μg sterol 10^8 cells), the data for HMSF134 showed FS/SE ratios of 9 and 12 in mid-log temperature-upshifted and non-temperature-shifted cultures, respectively (Table 2). Cells quantitated by dry weight showed FS/SE ratios of 10 and 12.5 when cultured under the same conditions (data not shown). The FS/SE ratios (mid-log, temperature upshifted) for X2180-1A, HMSF154, HMSF169 and HMSF190 ranged from 0.7 to 3.0 and were at least threefold lower than that for HMSF134 (Table 2). Similar values of FS/SE were obtained when dry weight determinations were used, 0.6 to 2.7 for all tested strains except HMSF134, which had an FS/SE ratio of 10 (data not shown).

Temperature profile of secretion mutant lipids. Analysis of the FS and SE by GLC revealed that when these strains were cultured between 26–34 C, the cells had measurable levels of SE and FS (Table 4). The maximum values for free sterol (2.8, 1.0, 0.9, 1.1 and 0.8 μg mg^{-1} dry wt) occurred between 30 C and 34 C and were for X2180-1A, HMSF134, HMSF154, HMSF169 and HMSF190, respectively. The FS/SE ratio for HMSF134

TABLE 2

Secretion Mutants and Parental Wild-Type Free and Esterified Sterol

	Free sterol (μg sterol 1 × 10^8 cells^{-1})				
	Wild-type X2180-1A	Vesicles[a] HMSF134	None[a] HMSF154	Golgi[a] HMSF169	ER[a] HMSF190
	Ratio free sterol (FS) to steryl ester (SE) (FS/SE)				
sta nts[b]	1.3	2.0	2.0	2.0	1.5
ml nts[c]	14.0	12.0	3.5	2.8	3.3
ml ts[d]	0.7	9.0	0.8	3.0	0.7

These cells were lyophilized and steamed in 100% dimethylsulfoxide. The results are from triplicate samples with less than 5% error.

[a]Accumulated structures on temperature upshift.

[b]sta nts—Stationary phase, no temperature shift.

[c]ml nts—Mid-exponential growth phase, no temperature shift.

[d]ml ts—Mid-exponential growth phase, temperature shift to 37 C.

TABLE 3

Total Sterol in Secretion Mutants and the Parental Wild-Type

	μg sterol 1 × 10^8 cells ml^{-1}				
	Wild-type X2180-1A	Vesicles[a] HMSF134	None[a] HMSF154	Golgi[a] HMSF169	ER[a] HMSF190
sta nts[b]	0.4	0.4	0.6	0.5	0.5
ml nts[c]	1.6	1.3	2.0	2.0	1.1
ml ts[d]	0.5	0.9	0.6	0.4	0.5

Total sterols from different classes of secretion mutants (μg sterol 1 × 10^8 cells^{-1}) obtained by alkaline pyrogallol saponification (12). The results are from triplicate samples with less than 4% error.

[a]Accumulated structures on temperature upshift.

[b]sta nts—Stationary phase, no temperature shift.

[c]ml nts—Mid-exponential growth phase, no temperature shift.

[d]ml ts—Mid-exponential growth phase, temperature shift to 37 C.

TABLE 4

Sterol Temperature Profile for Secretion Mutants and Parental Wild-Type

	Temperature (C)															
	26		28		30		32		34		36		38		40	
Strain	$\bar{X}$	± SD	$\bar{X}$	± SD	$\bar{X}$	± SD	$\bar{X}$	± SD	$\bar{X}$	± SD	$\bar{X}$	± SD	$\bar{X}$	± SD	$\bar{X}$	± SD
	Free sterol (μg sterol mg^{-1} dry weight)															
X2180-1A	1.7	0.5	2.3	0.1	2.8	0.1	3.2	0.4	2.3	0.1	1.4	0.1	1.0	0.1	0.5	0.1
HMSF134	0.6	0.1	0.6	0.1	0.6	0.3	0.7	0.2	1.0	0.1	nd[a]	nd	nd	nd	nd	nd
HMSF154	0.8	0.1	0.8	0.1	0.9	0.1	0.9	0.1	nd	nd	nd	nd	nd	nd	nd	nd
HMSF169	0.8	0.1	0.9	0.1	1.1	0.1	0.8	0.1	0.3	0.1	0.3	0.1	nd	nd	nd	nd
HMSF190	0.7	0.1	0.7	0.1	0.8	0.1	0.4	0.1	nd	nd	nd	nd	nd	nd	nd	nd
	Steryl ester (μg sterol mg^{-1} dry weight)															
X2180-1A	0.4	0.1	0.5	0.2	0.5	0.1	0.3	0.1	0.2	0.1	nde	nde[b]	nde	nde	nde	nde
HMSF134	0.6	0.1	0.9	0.1	0.8	0.1	1.3	0.4	1.1	0.2	nd	nd	nd	nd	nd	nd
HMSF154	0.7	0.1	0.7	0.2	1.0	0.2	1.2	0.1	nd	nd	nd	nd	nd	nd	nd	nd
HMSF169	0.6	0.1	1.4	0.1	1.3	0.1	1.1	0.1	0.4	0.1	nde	nde	nd	nd	nd	nd
HMSF190	2.0	0.1	2.3	0.1	2.1	0.1	1.3	0.1	nd	nd	nd	nd	nd	nd	nd	nd

Each strain was incubated in individual cultures over the temperature range of 26–40 C and harvested at 24 hr into stationary phase. Above the strain-specific restrictive temperature, these strains did not grow (nd). In the case of X2180-1A, free sterol was measured for the entire range of 26–40 C; however, steryl ester was not detected by GLC above 36 C (nde).

[a]nd: Not determined—cells did not grow at these temperatures.

[b]nde: No detectable ester.

observed under these conditions ranged from 1.0 to 0.9 for growth between 26 and 34 C, respectively. Analysis of total fatty acids from cells cultured to stationary (no temperature shift), mid-log (no temperature shift) and mid-log (with subsequent temperature shift) did not reveal any significant interstrain differences (data not shown), Comparative analyses of the fatty acids from cultures grown to stationary phase at different temperatures showed modifications to saturated and unsaturated fatty acid components (Figs. 2A-D). As the temperature increased (Figs. 2A and B), the content of 16:0 (except for HMSF190) and 18:0 (except in the wild-type) increased until a growth temperature just less than the restrictive temperature was reached. When the temperature was restrictive, there was a two- to threefold increase in the amount of 16:0 in the secretion mutant and wild-type (grew at 40 C) strains (Figs. 2A and B). For the 18:0 component, the increase was not as pronounced except for HMSF134. The 16:1 and 18:1 (except HMSF190) components decreased as the growth temperature increased, and when a growth temperature just below the restrictive temperature was reached, there was a large decrease in the amount of 16:1 and 18:1 (Figs. 2C and D). In each case, the sensitivity of the secretory mutants to temperature upshift was more pronounced than the wild-type.

Effect of temperature on secretion mutant plasma membranes. Analysis of the steady-state fluorescence anisotropy for plasma membrane preparations showed that in each strain—X2180-1A (wild-type parental), HMSF134, HMSF154, HMSF169 and HMSF190—there was a discontinuity in the plots for strains not subjected to a temperature upshift (Fig. 3). However, under conditions of temperature upshift, HMSF134 plasma membrane preparations showed no discontinuity, while the plots for X2180-1A, HMSF154, HMSF169 and HMSF190 displayed a discontinuity similar to that for non-temperature-upshifted cells (Fig. 4). In each case, except for HMSF134, the temperature at which the discontinuity in the fluorescence anisotropy plot appears is at a high temperature (3-6 C) for plasma membrane isolated from temperature-upshifted cells compared to plasma membrane from cells cultured at 24 C. The temperature at which these discontinuities appear (33-36 C in non-

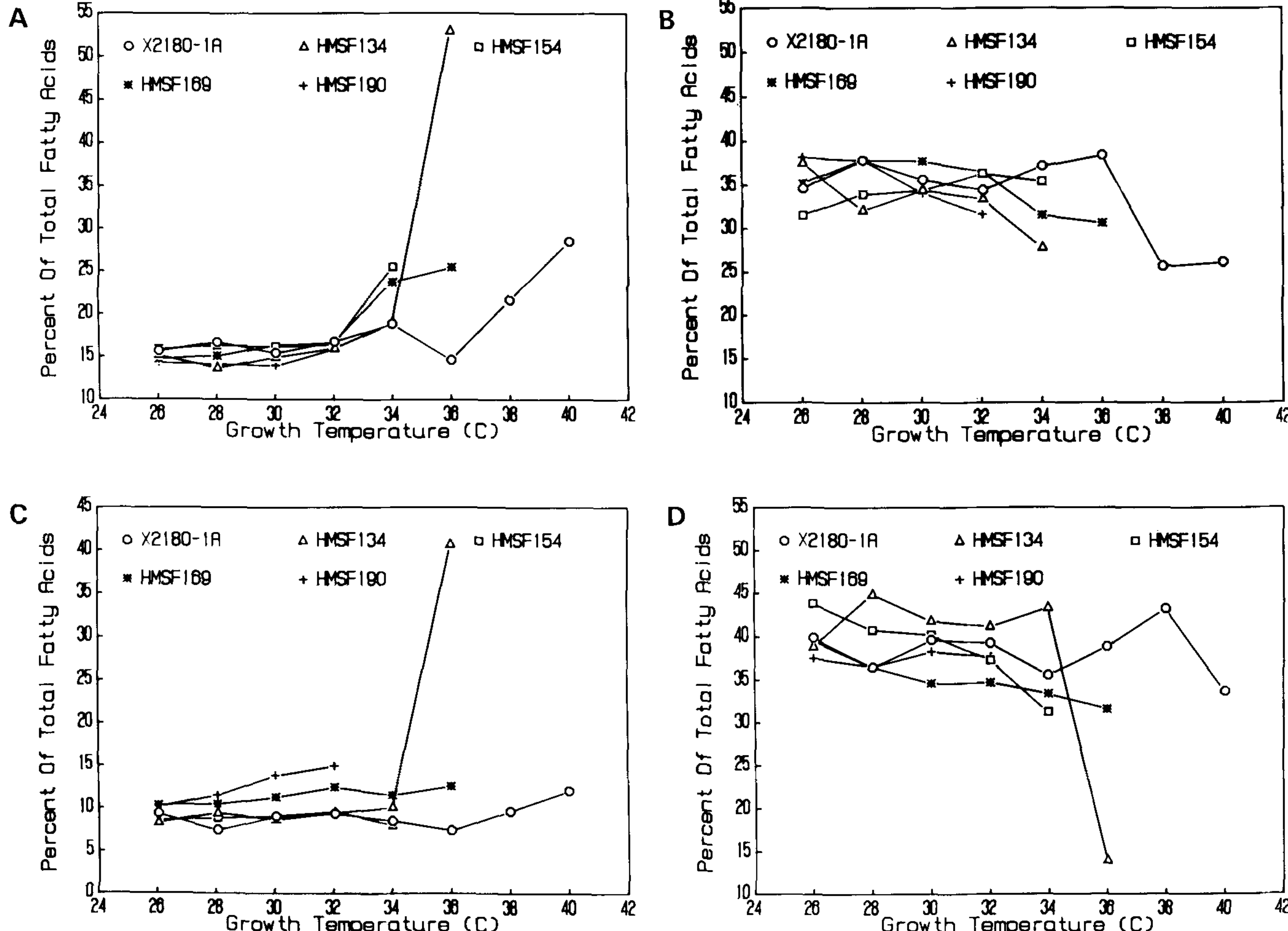

FIG. 2. Fatty acids in secretion mutants and the parental wild-type. Individual cultures were grown to stationary phase on yeast nitrogen base with dextrose over a temperature range of 26-40 C using a temperature gradient incubator. In Table 4, the temperatures above which these strains did not grow are indicated. For each long chain fatty acid species, the percent of the total long chain fatty acids is plotted (16:0, A; 18:0, B; 16:1, C; and 18:1, D).

temperature-shifted cells, and 36–42 C in temperature-upshifted cells) is above the optimal growth temperature range of 28–32 C for *S. cerevisiae*.

DISCUSSION

In yeast cells wild-type for ergosterol and sterol biosynthesis but conditionally defective for secretion properties, no large increase or decrease in total sterol content was observed in cells cultured to stationary phase (Table 3). There was, however, an increase followed by a decrease in intracellular FS and SE mg^{-1} dry wt (Table 4) as the growth temperature increased to 32 C. The observation that these maxima for FS and SE content occur at different temperatures in different strains may indicate a strain-related specificity for optimal lipid content as a function of growth temperature. Ramirez et al. have shown that under conditions of temperature stress, the yeast secretion mutants do not appreciably alter the phospholipid head group composition (10). Instead, we found that these yeasts modify the fatty acids attached to the membrane phospholipids. These changes in fatty acids reflect the increased sensitivity of the secretory mutants to thermal stress. The predominant changes appeared to be the fatty acids (Figs. 2A–D). The saturated fatty acids (16:0 and 18:0) increased while the unsaturated fatty acids either decreased (16:1) or remained unchanged (18:1). However, analyses of total fatty acids by chain length and unsturation showed larger fluctuations at a temperature just below the restrictive temperature in each different class of secretion mutant as well as the wild-type (X2180-1A). This may reflect cellular loss of regulatory function for membrane composition at that particular temperature. Based on the data presented here, modifying the fatty acid chain length and unsaturation appears to be the preferred method of maintaining plasma membrane integrity for growth at different temperatures when the sterol structure is fixed (yeasts wild-type for sterol synthesize only ergosterol as a normal end product). Comparing the total fatty acids (data not shown) with the sterol analyses (Table 4) indicated that the secretion mutants tested do not have an altered sterol biosynthetic metabolism that produces the membrane synthesis defect (cessation of membrane growth). Conceptually, this means that even when the yeast secretion mutants are conditionally blocked from new plasma membrane synthesis, they still produce lipid components that can potentially modify the plasma membrane for growth under temperature stress. These new plasma membrane precursors are not incorporated into existing plasma membrane when thermal stress is applied and the cell membrane ceases to grow. Therefore, when the plasma membrane preparations from temperature-stressed cells were examined, they reflected an increase in the temperature at which the discontinuity occurred. This is due to the inherent mixing of the existing plasma membrane and potential new membrane lipid components. This also would occur in the non-temperature-shifted cells, but since these cells were not temperature-stressed, the potential new membrane lipid components would not contain subunits optimal for growth at a higher temperature and would thus have a fluorescence anisotropy discontinuity at a lower temperature (Fig. 3).

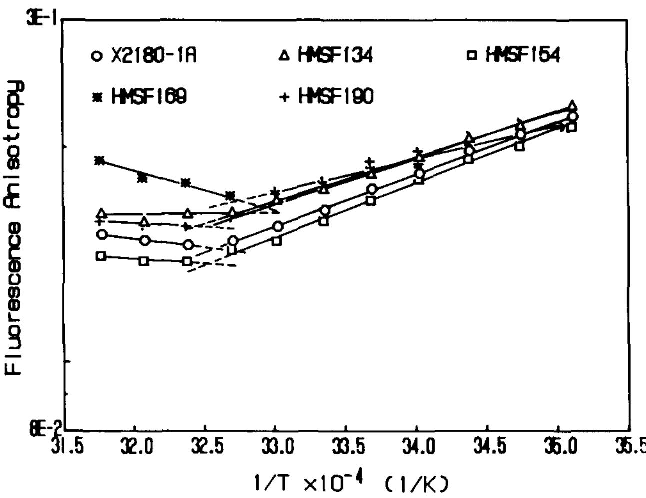

FIG. 3. Fluorescence anisotropy of plasma membrane from secretion mutants (24 C). Plasma membranes were isolated from secretion mutants cultured at 24 C to mid-log phase. The wildtype (X2180-1A) is shown for comparison over the range of 12 to 42 C.

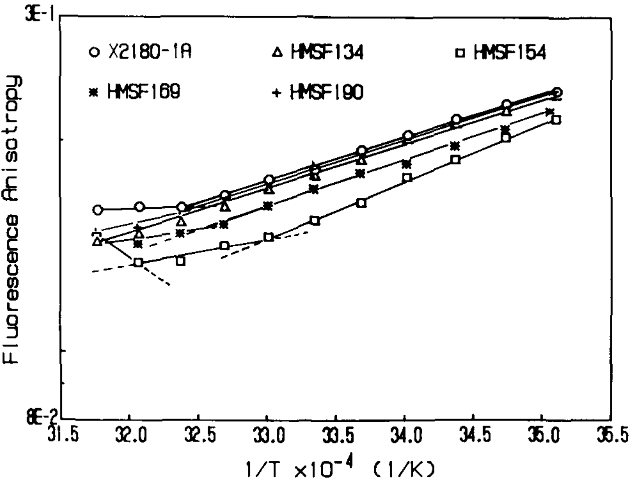

FIG. 4. Fluorescence anisotropy of plasma membrane from secretion mutants (37 C). Plasma membranes were isolated from secretion mutants cultured at 24 C to mid-log phase, then temperature upshifted to 37 C for 2 hr before harvesting. The wild-type (X2180-1A) is shown for comparison over the range of 12 to 42 C. For HMSF134, HMSF154, HMSF169 and HMSF190, the data points nearest the ordinal axis are almost coincident.

In the fluorescence anisotropy plot (Fig. 4), no discontinuity for HMSF134 was observed when the plasma membrane sample was from temperature-stressed cells, while in HMSF134 which had not been temperature-stressed, there was an observed discontinuity. The fact that no discontinuity was observed may be due to maintenance of a large FS pool rather than shunting FS into SE during temperature stress in this HMSF134 (Table 2). The vesicle membranes accumulated in HMSF134 may be enriched in FS that is not shunted into SE. Sterol enrichment of vesicle membrane (nascent plasma membrane at the permissive temperature) may produce larger fluctuations in fatty acid species to compensate for FS enrichment of nascent membrane. Inherent in the plasma

membrane preparation procedure is mixing of pre-existing and nascent (vesicle) plasma membrane. This mixing may produce a modified plasma membrane in which we observe the straight line plot for HMSF134. The mixing of pre-existing and nascent membrane is actually an advantage because it gives an overall distinction between pre-existing plasma membrane and nascent membrane optimized for growth at the permissive temperature, compared to preexisting plasma membrane (optimized for growth at 24 C) mixed with nascent plasma membrane optimized for the restrictive temperature. HMSF134, like the other strains tested, modifies the fatty acid content of its set of phospholipids (Fig. 2A–D). During a temperature upshift, the altered fatty acid composition and additional FS available may be sufficient to prevent loss of membrane integrity measured as a discontinuity in the fluorescence anisotropy plot. The observed discontinuities in the fluorescence anisotropy plots (Figs. 3 and 4) were determined by a least squares fit of the appropriate data points so that the calculated goodness of fit value (r) was >0.99.

ACKNOWLEDGMENTS

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Cyclic Fatty Esters: Hydroperoxides from Autoxidation of Methyl 9-(6-Propyl-3-Cyclohexenyl)-(Z)8-Nonenoate

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Autoxidation of the cyclic fatty acid ester, methyl 9-(6-propyl-3-cyclohexenyl)-(Z)8-nonenoate (I) was investigated to characterize the hydroperoxide isomers formed and to provide basic information on their chemistry, detection and effect on the quality of polyunsaturated cooking oils. Oxidation at 60 C with 1% hydroperoxide initiator produced a monohydroperoxide fraction containing five positional isomers (7-, 11-, 12-, 13- and 14-OOH), resolved by high performance liquid chromatography, as their allylic hydroxy esters. Their structures were established by ^{1}H- and ^{13}C-NMR spectroscopy and by capillary gas chromatography–mass spectrometry (GC-MS) as trimethylsilyl ether derivatives. Two additional isomers (8- and 9-OOH) were detected by GC-MS in only trace (<1%) quantities. Capillary GC resolved some geometric and stereoisomers, as well as positional isomers. Compared to photosensitized oxidation, two additional positional isomers (11- and 14-OOH) were produced by autoxidation. More stereoisomers were formed, and oxidation of the ring double bond was favored 8:1 over that of the side chain. This selectivity may be attributed to greater steric hindrance for oxygen attack at the side-chain double bond. A free radical mechanism is proposed to explain the greater isomeric complexity of the hydroperoxide products compared to photosensitized oxidation.
Lipids 22, 721–730 (1987).

Research on the autoxidation of fats is of worldwide interest because of nutritional, safety, storage and other economic problems resulting from the oxidative deterioration of fats, particularly those high in polyunsaturated fatty acids. Current understanding of the chemistry of autoxidation of fats has been reviewed recently in detail (1,2). When unsaturated fatty acids in vegetable oils undergo thermal oxidation in air, the primary oxidation products, monohydroperoxides, are formed relatively rapidly, and then react further to yield a variety of polar, secondary oxidation products, including dimers, polymers, volatile and nonvolatile degradation products (3,4).

One aspect of lipid research that has not received much attention is the oxidation of cyclic fatty acids (CFA). Methods are still needed to detect and characterize specific CFA and their reaction products. Previously, we synthesized and characterized a homologous series of CFA of known structures expected in heat-abused fats (5). We later prepared and characterized methyl 9-(6-propyl-3-cyclohexenyl)-(Z)8-nonenoate (I) and its hydrogenated derivatives (6), selected triglycerides (7) and primary photosensitized oxidation products (8). In this paper we report the isolation and characterization of the hydroperoxides formed by free radical autoxidation of the cyclic ester I.

MATERIALS AND METHODS

The preparation of cyclic fatty acid methyl ester (CFAME) I, the spectroscopic, nuclear magnetic resonance (NMR) and the chromatographic methods were essentially the same as described previously (8), except for the conditions for high performance liquid chromatography (HPLC), capillary GC, and GC-MS described below. Where necessary the NMR techniques of correlated spectroscopy (COSY) and selective homonuclear decoupling spectra were used to confirm structural assignments. COSY is a two-dimensional NMR technique used to acquire, in a single spectrum, information on those protons that are mutually coupled (9).

Autoxidation of I. CFAME I (2.0 g) was oxidized by heating isothermally at 60 or 80 C in a test tube with oxygen bubbling at a moderate rate from a capillary inlet tube. To initiate the autoxidation at 60 C it was necessary to add 1% of a hydroperoxide fraction previously isolated from the oxidation of CFAME at 80 C. Samples were analyzed periodically by thin layer chromatography (TLC) to follow oxidation. Autoxidized I was then fractionated by silicic acid chromatography (8), and the monohydroperoxides were further separated by preparative HPLC (10).

Silicic acid chromatography and HPLC of allylic hydroxy esters. The allylic hydroxy derivatives, produced by isolating the hydroperoxide fraction by silicic acid chromatography and reducing with $NaBH_4$, were separated by HPLC under the following conditions: Zorbax Sil preparative column (25 × 2.12 cm, i.d.; 6 μ silica); mobile phase, hexane/CH_2Cl_2/CH_3CN 5:3:0.25 by volume; flow, 4.0 ml/min at 1800 psi; infrared detector; sample injections, ca. 50–60 μl neat. Further separations were achieved by successive HPLC with Partisil M9 preparative column (100 × 0.94 cm, i.d.; 10 μ silica); mobile phase, hexane/CH_2Cl_2/EtOAc, 7:4:1 by vol; flow, 3.0 ml/min at 1100 psi; refractive index detector; sample injection, 16 mg/60 μl CH_2Cl_2. Further separations by HPLC were obtained by changing the mobile phase composition to 7:5:2, by reducing the flow to 1.5 ml/min and by using a Zorbax Sil analytical column (25 × 0.46 cm, i.d.; 5 μ particle size); mobile phase, hexane/CH_2Cl_2/EtOAc 7:4:1; flow, 2.0 ml/min at 600 psi; refractive index detector.

Capillary GC and GC-MS. Capillary GC was performed with a Perkin Elmer Sigma 3B gas chromatograph. Operating conditions were as follows: fused silica column (30 m × 0.24 mm i.d.) of SP2330 (0.2 μm film); linear gas velocity (He), 22.1 cm/sec; split ratio, 80/1; injector, 225 C; detector, 225 C. Column was heated isothermally at 170 C for 22 min and programmed at 10 C/min to 250 C. For GC-MS, a Finnigan MAT 1020/OWA system was used,

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Abbreviations: GC-MS, gas chromatography–mass spectrometry; CFA, cyclic fatty acids; CFAME, cyclic fatty acid methyl ester; NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; COSY, correlated spectroscopy; TLC, thin layer chromatography; TMS, trimethylsilyl ether; DEPT, distortionless enhancement by polarization transfer; EI, electron impact; IS, hydrogenated, saturated isomers of I.

scanning 23 AMU to 450 AMU in 2.0 sec, with the same conditions as used for analytical capillary GC.

RESULTS

Hydroperoxidation. When cyclic ester I was exposed to oxygen at 40 C, no oxidation was observed after 120 hr (see Table 1). At 60 C, oxidation produced only traces of hydroperoxides after 150 hr. However, in the presence of 1% hydroperoxide mixture isolated chromatographically from I as an initiator, autoxidation at 60 C resulted in 30% oxidation products after 80 hr. At 80 C, 18% oxidation occurred after 24 hr and 75% oxidation after 48 hr.

Hydroperoxides expected by the free radical autoxidation of cyclic ester I are shown in Figure 1. The 7-, 11-, 12-, 13- and 14-hydroperoxides were identified in significant amounts (normalized values: 4.2 to 38%) and the 8- and 9-hydroperoxides in minor amounts (0.4 to 1.9%). As in previous papers (5–8), we designate "*cis*" and "*trans*" for the relative geometry of the ring substituents (Δ10,15) of diene I or its derivatives, and "Z" and "E" for the relative geometry of substituents at double bonds of the side chain. Because diene I is initially a mixture of *cis* and *trans* isomers (ca. 25% and 75%, respectively), all hydroperoxides except the 8-OOH isomer consist of both *cis* and *trans* isomers. Because the 8-OOH isomer has a double bond between C-9 and C-10, there can no longer be any *cis/trans* relationship between substituents at C-10 and C-15. The allylic hydroxy esters formed by reducing

TABLE 1

Composition of Oxidation Products of Cyclic Diene I[a] by Silicic Acid Chromatography

Oxidation		Weight percent		
Temp (C)	Time (hr)	Hydroperoxides	Polar products	Unreacted diene I
40	120	0	0	100
60	150	tr	tr	100
60[b]	80[b]	17.5	11.9	70.6
80	24	14.0	3.7	82.2
80	48	28.7	45.8	25.5

[a]See Figure 1.
[b]Plus 1% hydroperoxide fraction from autoxidized diene I.

trans I (75%) + cis I (25%)

(a) −H•

A + B + C + D

(b) + $^3O_2$,H•

7-OOH (8.9%) + 8-OOH (1.9%) + 11-OOH (4.2%) + 12-OOH (37.8%)

+ 9-OOH (0.4%) + 10-OOH (0%)? + 13-OOH (32.5%) + 14-OOH (14.3%)

Where R = $(CH_2)_5$–C(=O)–OCH_3 (C-6 to C-1)

FIG. 1. Proposed free radical mechanism for hydroperoxide isomers formed by autoxidation of methyl 9-(6-propyl-3-cyclohexenyl)-(Z)8-nonenoate (I).

the hydroperoxides with $NaBH_4$ are designated as the 7-, 8-, 9-, 11-, 12-, 13- and 14-OH isomers. The saturated trimethylsilyl ether (TMS) derivatives formed by catalytic hydrogenation and silylation of the hydroperoxides or allylic hydroxy esters are designated as 7-, 8-, 9-, 11-, 12-, 13- and 14-TMS, respectively.

High performance liquid chromatography. The hydroperoxide fraction obtained by silicic acid chromatography of autoxidized cyclic ester I, proved to be a complex mixture of isomeric products that was too difficult to separate directly by HPLC. The HPLC separation was improved by reducing the hydroperoxides with $NaBH_4$ (Fig. 2). Major fractions 6–13 were shown by ^1H NMR to consist mainly of allylic hydroxy esters. Minor fractions 1–5 were mixtures of other components. Fractions 8 and 13 were further separated by HPLC under slightly different conditions (Figs. 3A and 4A). Fraction 8-2 was separated by HPLC into two more fractions, 8-2-1 and 8-2-2 (Fig. 3B), shown to consist of pure isomers by ^1H NMR. Fraction 13 (Fig. 3) was similarly resolved into additional components 13-1 and 13-2. Fraction 13-2 was resolved further into fractions 13-2-1, and 13-2-2 (Fig. 4), and they were characterized by ^1H and ^{13}C NMR, GC-MS, and capillary GC.

Nuclear magnetic resonance. Hydroperoxide components in main HPLC fractions (Figs. 2–4) were assigned one of the structures in Figure 1 (Tables 2 and 3) on the basis of ^1H, ^{13}C and distortionless-enhancement-by-polarization-transfer (DEPT) NMR spectra (see Tables 2 and 3). DEPT is a useful, time-saving technique for editing ^{13}C spectra into separate spectra for CH_3, CH_2 and CH groups (9). The ^{13}C NMR spectra provided additional information to identify specific isomers in Figure 1 with individual HPLC fractions. NMR spectra of the 7-, 9-, 12- and 13-OOH stereoisomers (Fig. 1) and their 9-, 12- and 13-OH allylic derivatives agreed with those reported previously (8). There was no evidence for any 8-OH or the 10-OH isomer in our NMR spectra of the major HPLC fractions 6–13.

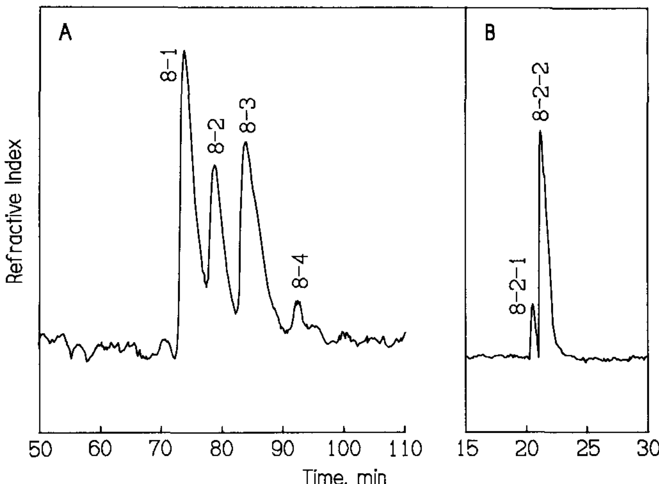

FIG. 3. Separation of $NaBH_4$-reduced hydroperoxides of fraction 8 (Fig. 2) by successive high performance liquid chromatography (HPLC). (A) Preparative column: 100 × 0.94 cm, i.d.; Partisil M9, 10 μ; mobile phase, hexane/CH_2Cl_2/EtOAc, 7:4:1, by vol; flow, 3.0 ml/min at 1100 psi. (B) Separation of fraction 8-2 by HPLC: mobile phase, hexane/CH_2Cl_2/EtOAc, 7:5:2 by vol as mobile phase; flow, 1.5 ml/min.

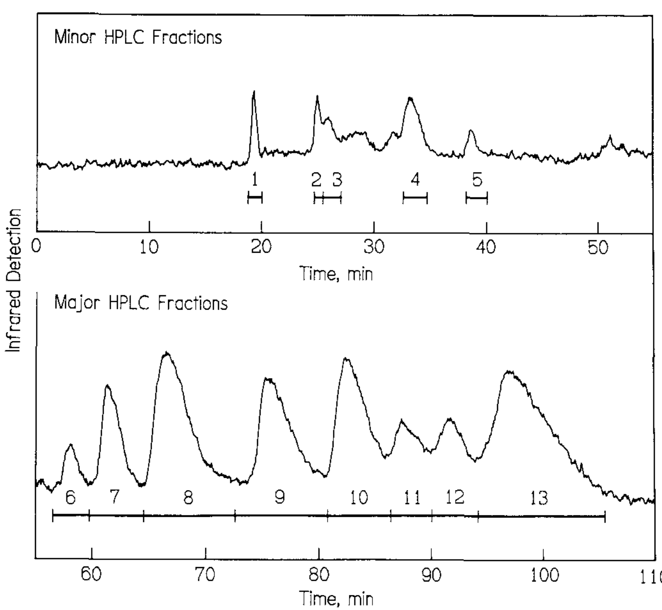

FIG. 2. Microporous silica high performance liquid chromatogram of $NaBH_4$-reduced hydroperoxides from autoxidation of cyclic ester I at 60 C for 80 hr. Preparative column: 25 × 2.12 cm, i.d.; Zorbax Sil, 6 μ; mobile phase, hexane/CH_2Cl_2/CH_3CN, 5:3:0.25 by vol; flow, 4.0 ml/min at 1800 psi.

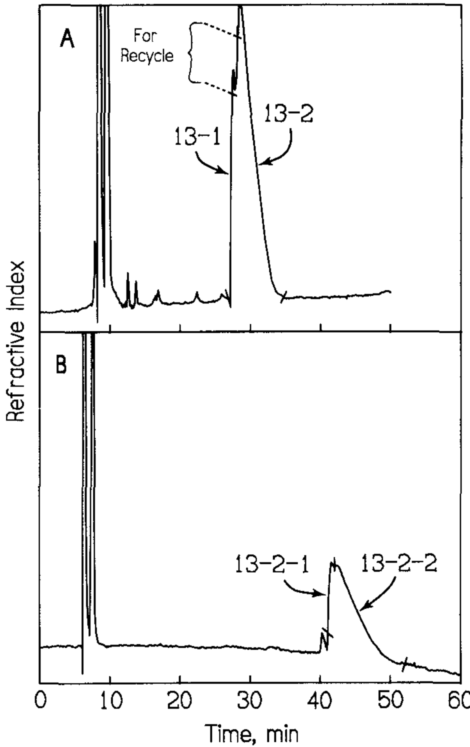

FIG. 4. Separation of components of fraction 13 (Fig. 2) by successive high performance liquid chromatography. (A) Same column and mobile phase as Fig. 3A. (B) Separation of fraction 13-2. Analytical column (25 × 0.46 cm, i.d.): Zorbax Sil, 5 μ; mobile phase, hexane/CH_2Cl_2/EtOAc 7:4:1; flow, 2.0 ml/min at 600 psi.

TABLE 2

^{1}H-NMR Data (300 MHz; $CDCl_3$) of HPLC Fractions[a] of Allylic Hydroxy Derivatives of Hydroperoxides[b]

Proton number	HPLC fraction (assigned structures)									
	7(7-OH)	11(7-OH)	8-3(11-OH)	9(12-OH)	10(13-OH)	12(13-OH)	13-1(13-OH)	6(14-OH)	8-1(14-OH)	8-2-2(14-OH)
H-7	4.38, $J_{7,8}$=8Hz	4.38, $J_{7,8}\sim$7Hz	2.08, $J_{7,8}$=7.3Hz	2.04m	2.03m	2.07m	2.07m	2.04, $J_{7,8}$=7.3Hz	2.02m	2.05
H-8	5.37, $J_{8,9}$=11.0Hz	5.34, $J_{8,9}$=11.0Hz	5.49, $J_{8,9}$=10.8Hz	5.37dt, $J_{8,9}$=10.9Hz	5.45m, $J_{8,9}$=10.8Hz	5.42m, $J_{8,9}$=10.7Hz	5.42dt, $J_{8,9}$=10.8Hz	5.39, $J_{8,9}$=10.9Hz	5.36dt, $J_{8,9}$=11.3Hz	5.39m
H-9	5.30, $J_{9,10}$=10Hz	5.39, $J_{9,10}$=7Hz	5.17, $J_{9,10}$=10.5Hz	5.16m, $J_{9,10}$=9.5Hz	5.11m, $J_{9,10}$=9.9Hz	5.11m, $J_{9,10}$=10.1Hz	5.20m, $J_{9,10}$=10.2Hz	5.14, $J_{9,10}$=9.9Hz	5.22m, $J_{9,10}$=10.0Hz	5.37m
H-10	2.36	2.36	2.71, $J_{10,11}$=3.2Hz	2.47m	2.67m	3.09m, $J_{10,11}$=5.1Hz	3.05m, $J_{10,11}$=4.9Hz	2.53	2.50m	2.91m
H-11	N.A.	N.A.	3.84, $J_{11,12}$=3.8Hz	1.70m	5.53m, $J_{10,11}$=2.3Hz	5.70dd, $J_{11,12}$=9.8Hz	5.57ddd, $J_{11,12}$=9.9Hz	2.06(a), 1.79(b)	1.90m	2.14m(a), 1.85m(b)
H-12	5.65m	5.64	5.79, $J_{12,13}$=9.9Hz	4.14m	5.80m, $J_{11,12}$=9.8Hz	5.78m	5.65m	N.A.	5.72m, $J_{12,13}$=10.0Hz	5.72m, $J_{12,13}$=10.0Hz
H-13	5.65m	5.64	5.89, $J_{13,14a}$=4.5; $J_{13,14b}$=2.5Hz	5.82br. s.	4.17m	4.16m	4.25m	N.A.	5.65m	5.80m
H-14	N.A.	N.A.	2.08(a), 1.76(b)	5.81br. s	1.93	1.65m	1.90m	4.12	4.03m	3.94m
H-15	N.A.	N.A.	1.91, $J_{10,15}$=3.2Hz	1.60	1.60	1.90m	1.69m	N.A.	1.40	1.59m

[a]See Figures 2–4.

[b]See Figure 1.

[c]Resonances common to all samples: δ0.87–0.91(t) for $-CH_3$ at C-18, δ2.29–2.31(t) for CH_2 at C-2, δ3.64–3.66(s) for $-OCH_3$ at C-1. Values expressed in ppm. Abbreviations: NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography; m, multiplet; t, triplet; d, doublet; s, singlet; br. s, broad singlet; N.A., no assignment.

TABLE 3

^{13}C NMR Data (75MHz; $CDCl_3$) of HPLC Fractions[a] of Allylic Hydroxy Derivatives of Hydroperoxides[b]

Carbon number	HPLC fractions (assigned structures)									
	7(7-OH)	11(7-OH)	8-3(11-OH)	9(12-OH)	10(13-OH)	12(13-OH)	13-1(13-OH)	6(14-OH)	8-1(14-OH)	8-2-2(14-OH)
C-6	37.5t	37.5t	29.0t	28.9t	29.0t	29.0t	29.0t	29.0t	29.0t	29.0t
C-7	68.0d	67.9d	27.8t	27.5t	27.6t	27.6t	27.6t	27.4t	27.5t	27.5t
C-8	132.1d	132.0d	127.2d[d]	130.3d	131.1d	127.4d[d]	130.5d[d]	130.1d[d]	129.9d[d]	129.5d
C-9	136.8d	137.2d	126.7d[d]	127.7d	128.1d	126.6d[d]	127.7d	128.6d	128.0d	128.4d
C-10	38.1d	38.0d	42.8d	36.6d	34.0d	31.4d	35.6d	32.1d	35.6d	31.2d
C-11	30.5t	30.5t	69.3d	38.1t	135.2d	134.6d	132.2d	30.8t	32.3t[e]	30.5t
C-12	126.7d[d]	126.6d[d]	131.5d[e]	63.9d	132.5d	131.3d	130.8d[d]	133.4d	133.0d	131.1d[d]
C-13	125.5d[d]	125.5d[d]	133.1d[e]	133.9d	64.2d	64.6d	68.4d	130.6d[d]	130.9d[d]	130.8d[d]
C-14	25.2t	25.2t	35.1t	135.0d	35.7t[d]	35.3t	35.9t	64.5d	71.0d	68.9d
C-15	37.2d	37.4d	31.8d	41.2d	40.7d	36.9d	36.7d	43.1d	46.3d	45.6d
C-16	32.4t	32.4d	35.1t	35.1t	35.8t[d]	33.8t	34.8t	35.6t	32.6t[e]	31.2t

Values expressed as ppm. NMR, nuclear magnetic resonance; HPLC, high performance liquid chromatography.

[a]See Figures 2–4.

[b]See Figure 1.

[c]Multipliciaties as follows: q, quartet; t, triplet; d, doublet; s, singlet. Resonances common to all samples were: δ174.3 ± 0.1(s) for C-1, δ34.1 ± 0.1(t) for C-2, δ24.8 ± 0.2(t) for C-3, δ14.4 ± 0.1(q) for C-18 and δ51.5 ± 0.1(q) for OCH_3. Resonances varied between isomers over a small range for C-4, δ28.6–29.7(t); C-5, δ25.0–29.4(t); C-16, δ29.5–35.5(t); and C-17, δ19.6–21.2(t).

[d]These assignments may be reversed.

[e]These assignments may be reversed.

Fractions 7 and 11 were shown to have a hydroxyl group at C-7 position, because coupling was observed between the proton on the hydroxyl-bearing carbon and the olefinic protons on carbons 8 and 9 (Table 2). For fraction 8-3, a hydroxyl group in the C-11 position was shown by 2D COSY and selective homonuclear decoupling experiments. The proton on the hydroxyl-bearing carbon was shown to be coupled to H-10, which in turn is coupled to H-9 and H-15 (see Table 2). Components of fractions 6, 8-1 and 8-2-2 were found to have the hydroxyl substituent on C-14. H-14 was coupled to the olefinic protons in the cyclohexene ring but not to H-10 (Table 2).

Slight variations in the proton chemical shifts for the same positional isomers in HPLC fractions of different retention volumes were considered as good evidence of stereoisomerism. For example, the H-10 shifts for the 14-OH isomer varied, from δ2.53 in fraction 6, δ2.50 in fraction 8-1 and δ2.91 in fraction 8-2-2 (Table 2), presumably due to the *cis* and *trans* configuration of side chains. Likewise, for the 13-OH stereoisomers of fractions 10, 12 and 13-1, the H-10 shifts were at δ2.67, δ3.09 and δ3.05, respectively. However, no variation of H-10 shifts (or H-12 or H-13) was observed between 7-OH stereoisomers of fractions 7 and 11, apparently because the OH substituent is relatively less influenced by configurational and conformational differences of the cyclohexene moiety in the 7-OH isomer than in the 11-, 12-, 13- and 14-OH isomers. A comparison of the chemical shifts for carbons as well as protons showed that fractions 10 and 12 (Table 2) correspond to the same 13-OH stereoisomers as previously obtained by photosensitized oxidation of I (8). However, fraction 13-1 corresponds to a different 13-OH stereoisomer. Also, when the 12-OH stereoisomer of fraction 9 (Table 2) was compared with the 12-OH stereoisomer isolated from photosensitized oxidation (8), no differences in these chemical shifts or coupling constants were detected, indicating that the same 12-OH stereoisomer was present in both sources.

Because all of the olefinic protons listed in Table 2 exhibited a vicinal coupling constant of less than 12 Hz, all isomers isolated and identified here by NMR have only Z-double bonds. However, the 7-OH isomer isolated previously from the photosensitized oxidation of I contained an E-double bond at C-8, as evidenced by its coupling constant $J_{7,8}$ = 15.5 Hz (8).

The ^{13}C NMR data (Table 3) were consistent with the ^{1}H NMR data (Table 2) in supporting the structures for the hydroperoxide components, based on previously established assignments (8). Multiplicities of signals were determined by DEPT experiments, and the expected resonances for the side chain propyl and methyl ester groups were observed. Although resonances attributed to C4-6 and C-17 varied between isomers, they were not especially helpful in distinguishing between isomers. Variations in shifts of C-15 and C-16 were due to either *cis* or *trans* ring substitutions. ^{13}C NMR assignments for the olefinic, methinyl and hydroxyl-bearing carbons (C-7 to C-14; Table 3) were most helpful in determining positional isomers. Slight variations in the chemical shifts of C7-14 were observed between stereoisomers in spectra of HPLC fractions 10, 12 and 13-1. Fraction 10 had the same ^{13}C chemical shifts as the previously reported 13-OH stereoisomer (8).

GC-MS characterizations. Characteristic MS and interpretations of the electron-impact (EI) MS fragmentations for the positional isomers 7-, 8-, 9-, 12- and 13-TMS were reported in our previous investigation of the photooxidation of cyclic ester I (8). The 11- and 14-TMS isomers were characterized by MS because they are derived from unique hydroperoxides of the autoxidation of I (Fig. 1). The cyclohexanol cleavage mechanism was previously invoked to explain the fragmentation of isomers 13-TMS

FIG. 5. Proposed electron impact-mass spectrometry fragmentations for hydrogenated trimethylsilyl ether derivatives of: (A) 11- and 14-OOH isomers and (B) 10-OOH isomer (Fig. 1).

and 12-TMS, giving the characteristic masses of the 13-TMS (m/z = 171) and 12-TMS (m/z = 299) isomers for selected ion monitoring (11). However, the cyclohexanol cleavage of isomeric 11-, 14- and 10-TMS derivatives produces only common mass fragments (Fig. 5). The favored mass fragment m/z 129 predicted by this cleavage mechanism was also relatively abundant in the EI-MS spectra of the other positional isomers, 7-, 8-, 9-, 12- and 13-TMS. Any other characteristic mass fragments were either too weak to detect or absent in the EI-MS of HPLC fractions 6, 8-2, 8-3 and 8-4, composed of either 11- or 14-TMS (Table 4). Likewise, on the basis of the cyclohexanol cleavage mechanism (Fig. 5B), EI-MS of 10-TMS isomer was not expected to yield a characteristic mass useful for mass chromatography, because the favored mass fragment m/z = 299 is also produced by isomer 12-TMS (8). The allylic 10-OH isomer was not detected by NMR in any reduced HPLC fractions isolated from either photooxidation (8) or autoxidation products of I.

The hydroperoxide components of fractions 6, 8-2-2 and 8-4 (Figs. 2–4) were all shown to have essentially the same skeletal structure by MS. Fraction 6 gave the following representative mass fragments (after hydrogenation and silylation), m/z (rel int): M-15, 369 (2.0); M-31, 353 (1.9); 341 (2.3); 337 (7.5); M-90, 294 (24); 252 (9.0); 171 (4.6); 159 (7.8); 151 (11.5); 138 (17.3); 129 (50); B, 73 (100); 43 (27). These results together with NMR spectra show that these fractions contain methyl 14-hydroxy-9(2-propylcyclohexyl) nonanoate (as 14-TMS). Similarly, EI-MS of fraction 8-3 gave the following representative fragments (after hydrogenation and silylation), m/z (rel int): M-15, 369 (1.7); M-31, 353 (0.9); 341 (4.2); M-90, 294 (19.2); 285 (0.9); 251 (7.4); 171 (9.3); 159 (7.1); 151 (4.1); 143 (8); 137 (13.4); 129 (46.3); 81 (44.1); B, 73 (100); 43 (26.5). These data together with NMR spectra are consistent with the methyl 11-hydroxy-9-(2-propylcyclohexyl)nonanoate (as 11-TMS), derived from the 11-OOH isomer.

Hydroperoxides present in the minor HPLC fractions 1–5 (Fig. 2) could be identified by GC-MS and capillary GC retention. GC-MS showed that ca. 81% of fraction 1 was unreacted I (Table 4). About 44% of fraction 2 consisted of 8-TMS isomers, but >50% could not be identified. Approximately 30% of fraction 4 was due to 13-TMS isomers and 16% to 12-TMS isomers. Fractions 3 and 5 were not identified. The 9-TMS derivative was only detected in fractions 2 and 5 and represented only ca. 2–3% and ca. 4%, respectively.

Capillary GC. Different positional isomers have geometric or stereoisomers with identical or nearly identical relative retentions (Table 4). These relative retentions may be shifted slightly by variations in the sample size or composition, and this type of evidence alone cannot be relied upon to positively identify positional isomers, especially when particular stereoisomers of different positional isomers showed differences of only 0.02 units or less in relative retentions. Therefore, confirmation of their identity by mass chromatography was required.

The isomeric composition of the isolated hydroperoxide fraction was determined quantitatively by capillary GC of the saturated TMS derivatives (Table 5, Fig. 6). About 19% unidentified material eluted after the TMS

TABLE 4

Identification of Trimethylsilyl Ether (TMS) Derivatives of Hydroperoxides of I[a] in HPLC Fractions by Capillary GLC and GC-MS

HPLC fraction (Figs. 2–4)	Capillary GC/GC-MS[b] of hydrogenated, silylated fraction			
	Compound	Relative retention (R_r)	Relative percent[c]	Characteristic mass
Minor fractions 1	*trans* IS	1.00	64.3	296
	cis IS	1.08	16.4	296
	Unidentified		19.3	
2	8-TMS	2.59	35.2	245
	8-TMS	1.64	3.4	245
	8-TMS	2.07	1.7	245
	8-TMS	2.92	4.1	245
	9-TMS	1.50	1.3	259
	9-TMS	2.66	1.3	259
	Unidentified		53.0	
3	8-TMS	2.55	6.2	245
	Unidentified		93.8	
4	12-TMS	1.40	7.5	299
	12-TMS	1.93	3.5	299
	12-TMS	2.97	5.3	299
	13-TMS	1.49	8.7	171
	13-TMS	1.90	1.3	171
	13-TMS	2.09	3.4	171
	13-TMS	2.65	8.4	171
	13-TMS	2.75	3.1	171
	13-TMS	3.03	5.2	171
	Unidentified		53.6	
5	9-TMS	1.19	4.2	259
	Unidentified		95.8	
Major fractions 6	*trans* IS	1.00	39.9	296
	cis IS	1.07	2.1	296
	9-TMS	1.18	3.2	259
	trans 14-TMS	1.37	47.7	—
	Unidentified		7.1	
7	*trans* IS	1.00	44.8	296
	cis IS	1.08	14.9	296
	trans 7-TMS	1.38	27.1	231
	cis 7-TMS	1.45	6.1	231
	Unidentified		7.0	
8-1	*trans* IS	1.00	21.5	296
	cis IS	1.08	8.8	296
	13-TMS	1.27	5.6	171
	trans 7-TMS	1.39	45.7	231
	cis 7-TMS	1.45	7.3	231
	Unidentified		11.1	
8-2-2	*trans* IS	1.00	0.3	296
	cis IS	1.08	3.5	296
	trans 14-TMS	1.27	5.4	—
	cis 14-TMS	1.35	85.8	—
	Unidentified		5.0	
8-3	*trans* IS	1.00	1.3	296
	cis IS	1.08	51.0	296
	cis 11-TMS	1.29	39.0	—
	11-TMS	1.34	3.5	—
	11-TMS	1.43	2.3	—
	Unidentified		2.9	
8-4	*trans* IS	1.00	15.9	296
	cis IS	1.08	1.1	296
	trans 14-TMS	1.69	77.1	—
	Unidentified		5.9	
9	*trans* IS	1.00	65.1	296
	cis IS	1.07	4.1	296
	trans 12-TMS	1.40	27.1	299
	Unidentified		3.7	
10	*trans* IS	1.00	40.2	296
	cis IS	1.07	7.7	296
	7-TMS	1.38		231
	12-TMS	1.39	4.1u	299
	trans 13-TMS	1.50	40.4	171
	12-TMS	1.94	4.8	299
	Unidentified		2.8	
11	*trans* IS	1.00	45.6	296
	cis IS	1.08	10.8	296
	trans 7-TMS	1.39	28.1	231
	cis 7-TMS	1.44	4.9	231
	13-TMS	1.48	2.5	171
	8-TMS	1.82	1.1	245
	13-TMS	1.92	1.6	171
	Unidentified		5.4	
12	*trans* IS	1.00	6.3	296
	cis IS	1.08	32.4	296
	7-TMS	1.45	1.8	231
	13-TMS	1.49	1.2	171
	cis 13-TMS	1.84	44.7	171
	13-TMS	2.04	2.6	171
	13-TMS	2.18	2.5	171
	Unidentified		8.5	
13-1	*trans* IS	1.00	2.9	296
	cis IS	1.08	27.4	296
	12-TMS	1.71	3.9	299
	trans 13-TMS	1.93	4.4	171
	cis 13-TMS	2.02	53.7	171
	Unidentified		7.7	
13-2-1	*cis* 12-TMS	1.69	25.7	299
	trans 13-TMS	2.02	74.3	171
13-2-2	*trans* IS	1.00	21.0	296
	cis IS	1.08	1.9	296
	12-TMS	1.71	6.3	299
	trans 12-TMS	1.88	49.0	299
	12-TMS	1.94	1.9	299
	13-TMS	2.05	15.5	171
	Unidentified		4.4	

HPLC, high performance liquid chromatography; GLC, gas liquid chromatography; GC, gas chromatography; MS, mass spectrometry.

[a]See Figure 1.

[b]See Materials and Methods.

[c]The *cis* and *trans* saturated isomers of I(IS) were produced by catalytic hydrogenolysis (see text); u = unresolved peaks (7-TMS and 12-TMS).

TABLE 5

Capillary GC[a] of Hydroperoxide Fraction[b] of Autoxidized CFAME I[c] After Hydrogenation and Silylation

Peak(s) (see Fig. 6)	Assignment[d]	Relative retention (R_r)	Relative percent	
			With I	Without I[e]
1–3	?	0.33–0.48	0.47	0.50
4	*trans* I	1.00[f]	5.07	0.00
5	*cis* I	1.08	1.65	0.00
6	9-OOH	1.15	0.25	0.27
7	?	1.24	0.48	0.51
8	14-OOH	1.27	4.03	4.32
9	11-OOH	1.29	0.15	0.16
10	11-OOH	1.33	3.01	3.23
11	14-OOH	1.36	1.82	1.95
12	7-OOH	1.38	3.38u	3.62u
13	12-OOH	1.40	17.27	18.52
14	7-OOH	1.45	3.29	3.53
15	13-OOH	1.49	9.14	9.80
16	8-OOH[g]	1.60	0.21	0.23
17	14-OOH	1.67	4.99u	5.35u
18	12-OOH	1.71	2.95	3.16
19	8-OOH[g]	1.82	0.39	0.42
20	12-OOH	1.86	8.28	8.88
21	13-OOH	1.93	5.92	6.35
22	13-OOH	2.01	3.53u	3.78u
23	13-OOH	2.05	5.55	5.95
24	8-OOH[g]	2.50	0.22	0.24
25	8-OOH[g]	2.53	0.13	0.14
26	8-OOH[g]	2.56	0.14	0.15
27	8-OOH[g]	2.59	0.08	0.09
28	13-OOH	2.74	0.19	0.20u
29	13-OOH	2.75	0.23	0.25
30–32	?	2.78–2.86	2.40	2.56
33	?	2.90	0.26u	0.28
34	8-OOH[g]	2.93	0.19	0.20
35	?	2.99	0.32	0.34
36–47	?	3.04–3.84	13.99	15.02
			99.98	100.00

GC, gas chromatography; CFAME, cyclic fatty acid methyl ester; MS, mass spectrometry.

[a]See Materials and Methods.

[b]Fraction isolated by silicic acid chromatography (10).

[c]Methyl 9-(6-propyl-3-cyclohexenyl)-(Z)8-nonenoate oxidized at 60 C for 80 hr (see Table 1). *Trans* and *cis* refer to ring substituents at C-10 and C-15.

[d]Identity as hydroperoxide precursor or diene I isomers, based on previous GC-MS, mass chromatography and nuclear magnetic resonance correlations.

[e]Normalized after excluding I (*cis* and *trans*). u = Unresolved peak.

[f]Retention time (t_r) = 10.00 min.

[g]Tentative assignments, based on previous relative retentions (Table 4).

derivatives. Capillary GC analyses indicated that initially the major portion (ca. 57%) consisted of 26.3% 13-OOH and 30.6% 12-OOH. A smaller portion (23% or less) consisted of 3.4% 11-OOH, 11.6% 14-OOH, 7.2% 7-OOH and 1.5% 8-OOH. Only a trace (ca. 0.3%) of 9-OOH was identified in the primary oxidation fraction. Thus, unlike photooxidation at 0 C (8), autoxidation of I at 60 C favored the formation of hydroperoxides from the ring double bond by a ratio of 8:1 over the side-chain double bond of I.

When the HPLC fractions containing allylic OH esters were hydrogenated, saturated isomers of I (defined as IS) were formed. The *trans* and *cis* saturated isomers of rel retention 1.00 and 1.08, respectively, were detected by capillary GC and GC-MS (Table 4). The occurrence of these geometric isomers is attributed to catalytic hydrogenolysis of the allylic hydroxy esters followed by their hydrogenation (12). Because the IS isomers were formed by hydrogenation only, they were not present in the initial HPLC fractions analyzed by NMR. However, the fortuitous formation of *cis* and *trans* IS isomers in the hydrogenated fractions allowed us to calculate the relative retention times of the corresponding TMS isomers. Geometric TMS isomers were tentatively assigned, therefore, on the basis of the respective ratio of the IS peaks. For example, in fraction 6, GC showed 39.9% *trans* IS but only 2.1% *cis* IS with only one major peak (47.7%) corresponding to *trans* 14-TMS (R_r = 1.37). Therefore, the tentative assignment of *trans* and *cis* 13-TMS isomers with characteristic m/z = 171 (8) was made on the basis of the observed ratio of the respective IS isomers.

DISCUSSION

Cyclic fatty acids are generally considered to be formed in only trace amounts in heated fats. However, recent analyses revealed monomeric CFA at levels ranging from 0.1% to 0.5% in commercial frying oils in the U.S. (13). Therefore, little or no oxidation of CFA might be expected in the presence of the usual amounts of linoleate and linolenate in vegetable oils. Concentrated mixtures of CFA isolated from severely thermally abused oils were found to be toxic when fed experimentally to animals in high doses (14–18). However, because these feeding studies were generally conducted only with mixtures of CFA, those chemical structures responsible for the observed toxicity have not yet been determined (17–19).

In the photosensitized oxidation of diene I, concerted addition of singlet oxygen and double bond migration produced the (E)7-, 8-, (E)9-, 12- and 13-hydroperoxide (OOH) isomers (14). The 7-, 10-, 11- and 14-OOH isomers may be formed from the 8-, 9-, 12- and 13-OOH by allylic rearrangement (8). However, by free radical autoxidation, the 7-, 10-, 11- and 14-OOH positional isomers (see Fig. 1) are expected to be formed as primary products directly from I. All positional isomers of the hydroperoxides of Figure 1 have three stereocenters and 2^3 possible stereoisomers, except the 8-OOH and 10-OOH isomers. The latter have only two stereocenters and 2^2 possible stereoisomers. Many of these stereoisomers were partially resolved by capillary GC. The hydroperoxide mixtures characterized in this study are more complex than the hydroperoxides from photosensitized oxidation of cyclic ester I (8), because positional, geometric (E-,Z- and Z-,Z-) and stereoisomers are formed in unequal proportions. The autoxidation of I produced the 11- and 14-OOH isomers not previously found in photosensitized oxidation (8). The presence of these new hydroperoxide isomers supports the free-radical mechanism shown in Figure 1.

The different distribution of hydroperoxide isomers summarized in Figure 1 can be attributed to steric hindrance from the side chains of the cyclohexene in I. Oxygen attack at the end carbons of the allylic free

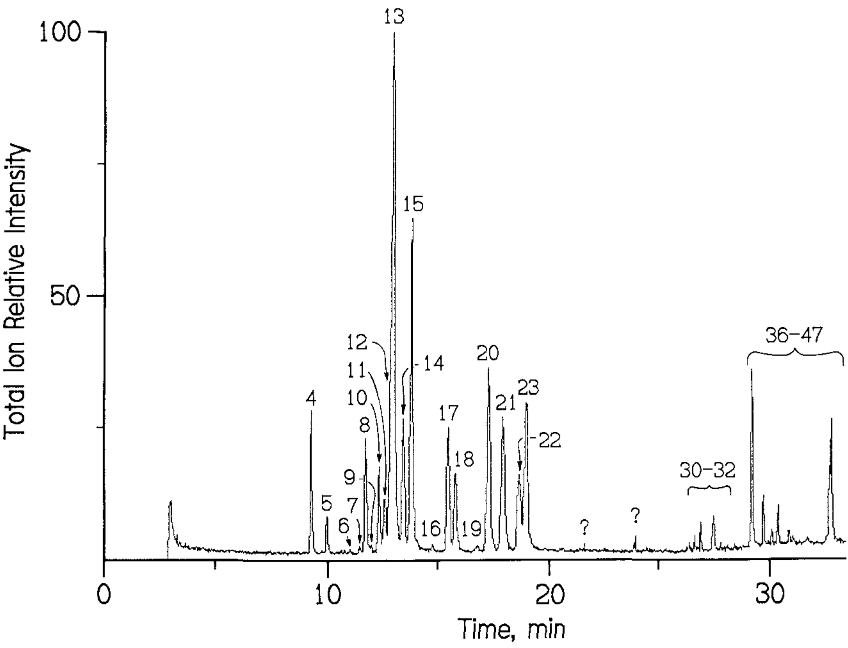

FIG. 6. Capillary gas chromatography-mass spectrometry fragmentations for hydrogenated trimethylsilyl ether derivatives of: (A) 11- and 14-OOH isomers and (B) 10-OOH isomer (Fig. 1).

FIG. 7. Alternate free radical cyclization mechanism.

radicals is hindered in varying degrees by the ester and propyl side chains. Formation of the 12- and 13-OOH isomers is most favored, because the 12- and 13-carbons are farthest from the side chains and least hindered. The longer and bulkier ester side chain exerts more steric hindrance at an adjacent carbon than the shorter propyl group. Therefore, the 14-OOH isomer is formed in larger amount than the 11-OOH isomer. Likewise, because carbon-7 is more accessible than carbon-9, more 7-OOH isomer is formed than 9-OOH isomer. The 9- and 10-carbons appear least accessible to oxygen attack. The 9-OOH isomer is formed in only trace amounts, and the absence of 10-OOH isomer can be explained by the inaccessibility of the trisubstituted carbon-10 to oxygen

attack. The intermediate allylic radical B may not be formed because of this steric hindrance.

An alternative mechanism to explain the absence of the 10-OOH isomer and the relatively low concentration of the 11-OOH isomer may involve the homo-allylic double bond leading to 1,3-cyclization of their peroxyl radicals (Fig. 7). This type of cyclization was proposed for the formation of bicycloendoperoxides from polyunsaturated fatty acids containing three or more double bonds (20). Similar five-membered hydroperoxy epidioxides were identified in autoxidized methyl linolenate (10,21,22). These secondary oxidation products are formed by cyclization of the 12- and 13-hydroperoxyl radicals of linolenate. This reaction accounts for the concentrations of the internal 12- and 13-hydroperoxide isomers being lower than the external 9- and 16-OOH isomers, a well-known characteristic of linolenate autoxidation (1,2). However, the cyclization mechanism of Figure 7 needs to be substantiated by further characterization of the secondary oxidation products of I.

The present work reports the successful application of high resolution NMR and mass spectrometry to the characterization of hydroperoxides formed from the autoxidation of a CFA that contains a center of unsaturation both in a six-membered ring and in a chain substituent. By this approach, the relative oxidative susceptibility of each type of double bond was determined.

Our results also indicate that pure ester I or its CFA would undergo autoxidation even at 60 C if initiated by other hydroperoxides, to generate a complex mixture of isomeric cyclic hydroperoxides. This hydroperoxide mixture may readily undergo secondary oxidations to form condensation and decomposition products. This information should aid other investigators in analyzing, identifying and isolating CFA hydroperoxides in heated fats and in evaluating their effects on the quality and nutritional deterioration of polyunsaturated fats.

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Characterization of Phospholipase A_2 from Rabbit Lung Microsomes[1]

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A phospholipase A_2 activity associated with the microsomal fraction of rabbit lung homogenates was studied. The enzyme showed specificity for the *sn*-2 ester bond of phosphatidylcholine, had an alkaline pH optimum and required Ca^{2+} for activity. Other divalent cations were unable to support hydrolysis. In the absence of detergents, exogenous phosphatidylethanolamine was deacylated at a rate sevenfold higher than phosphatidylcholine. The activity toward both substrates could be enhanced by sodium deoxycholate or, more effectively, by sodium taurodeoxycholate. Phosphatidylethanolamine required higher detergent/phospholipid molar ratios than phosphatidylcholine. Under these conditions, the preference for the former substrate over the latter was nearly abolished. The zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and the nonionic detergent Triton X-100 were either ineffective (phosphatidylcholine) or inhibitory (phosphatidylethanolamine). Addition of KCl produced opposite effects on the activity depending on the bile salt used to disperse the substrate. The phospholipase A_2 activity was inhibited by *p*-bromophenacyl bromide but remained unaffected after treatment with diisopropylfluorophosphate or NaF. N-Ethylmaleimide, but not other thiol reagents, partially inhibited the activity.
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Phospholipase A_2 (EC 3.1.1.4) catalyzes the hydrolytic removal of a fatty acyl chain from the *sn*-2 position of phospholipids. While secretory phospholipases A_2 such as those from the venoms of serpents and arthropods and from pancreatic juice have been studied in great detail (1), much less is known about their intracellular counterparts, including their exact physiological function(s) (2). A distinctive characteristic of the latter group of phospholipases is their relatively low activity under the experimental conditions habitually used (1,2).

The presence of phospholipases in lung tissue is well documented. Following the initial observations made with whole homogenates (3-6), Otha et al. (7) described a Ca^{2+}-independent phospholipase A_2 in rat lung high-speed supernatant that was inhibited by fluoride and by sodium deoxycholate. A second Ca^{2+}-independent activity, specific for 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor), was later reported in lung cytosol (8). Recently these two phospholipases have been separated not only from each other but also from yet a third cytosolic, Ca^{2+}-dependent phospholipase A_2 (9). Garcia and coworkers showed that while rat lung mitochondria and microsomes both contained phospholipase A_2 (10), no activity was associated with the lamellar bodies (11). These findings were confirmed for microsomes and lamellar bodies from rat lung by Longmore et al. (12). However, lamellar bodies from rabbit lungs were reported to express phospholipases A_1 and A_2 (13). In lung homogenates from rabbits and dogs, phospholipase A_2 was demonstrated in nearly all subcellular fractions (14).

We decided to study phospholipase A_2 in lung microsomes because of the generally accepted involvement of these membranes in phospholipid biosynthesis (15,16) and the reported absence of appreciable phospholipase A_1 in lung microsomal preparations (10,12,17). In the present work, we confirm the specificity of the enzyme[2] for the *sn*-2 position of phosphatidylcholine and describe the optimal conditions for its assay with exogenous substrates as well as the effects of detergents, salt, inhibitors and divalent cations.

MATERIALS AND METHODS

Materials. Water was deionized through a Milli-Q Reagent Water System (Millipore, Mississauga, ON, Canada). Radioactive substrates were from NEN (DuPont, Montreal, PQ, Canada). Phosphatidylethanolamine, obtained from egg phosphatidylcholine by transphosphatidylation, and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine were from Avanti Polar Lipids (Birmingham, AL). Dipalmitoylphosphatidylcholine, sodium deoxycholate, sodium taurodeoxycholate, diisopropylfluorophosphate and *p*-hydroxymercuribenzoate were from Sigma Chemical Co. (St. Louis, MO). *p*-Bromophenacyl bromide was from Aldrich (Milwaukee, WI). 3-[(3-cholamidopropyldimethylammonio]-1-propanesulfonate (CHAPS) and Triton X-100 were purchased, respectively, from Boehringer Mannheim (Dorval, PQ, Canada) and J.T. Baker (Phillipsburg, NJ). LK6D thin-layer plates were from Whatman (Clifton, NJ). All other reagents were from BDH (Toronto, ON, Canada).

Isolation of microsomes. Pregnant New Zealand White does of 24 days gestation whose fetuses were used in other experiments were killed by intravenous injection of pentobarbital, and their lungs were excised. After being washed the lungs were briefly disrupted in a Waring blender and homogenized in a Potter-Elvehjem tube in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCl (pH 7.4) to yield a 10% (w/v) homogenate. After centrifugation at 1,000 × g for 5 min, the resulting supernatant was centrifuged at 20,000 × g for 20 min. The supernatant obtained from the latter centrifugation was then subjected to centrifugation at

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Abbreviation: CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate.

[2]The word "enzyme" is used throughout for the sake of simplicity. Especially because lung microsomal preparations result from a heterogeneous population of cells, the possibility exists that such preparations contain different proteins with phospholipase A_2 activity.

105,000 × g for 1 hr. The resulting pellet (henceforth referred to as microsomes) was then gently resuspended in the same buffer at an approximate protein concentration of 10 mg/ml, immediately frozen and kept at -80 C until use.

Phospholipase A_2 assay. Unless indicated otherwise in figure legends, the standard incubation mixtures (final volume 0.2 ml), made up in 100 mM Tris-HCl (pH 8.5), contained 20 nmol of EDTA, 2 μmol of $CaCl_2$, 1.5 μmol of sodium taurodeoxycholate and 1.6 μmol of 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (specific radioactivity 300–400 dpm/ nmol). In some cases, 1.6 μmol of a mixture of 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoethanolamine and unlabeled phosphatidylethanolamine (see *Materials*) to give a specific radioactivity of 300-400 dpm/ nmol was used under conditions described in the figure legends. The reaction was started by the addition of 80 μg of microsomal protein (corresponding to ca. 65 nmol of membrane phospholipid) and incubated at 37 C. After 1 hr, the volume was adjusted to 0.5 ml with water, and the mixture was subjected to a modified Dole extraction, essentially as described by van den Bosch and Aarsman (18). Blanks without microsomes were always included, and the results were corrected for the background radioactivity thus extracted (less than 0.05% of the total radioactivity of the assay mixture even when detergents were present).

Except for the positional specificity of the enzyme (Fig. 2), which was determined only once in duplicate, the results are representative of at least two similar experiments carried out with different microsomal preparations. In all cases, each data point is the average of duplicate determinations whose average deviation from the mean was less than 8%.

Analytical techniques. Protein was determined by the method of Lowry et al. (19) using bovine serum albumin as standard.

RESULTS

The optimal conditions for lung microsomal phospholipase A_2 activity toward exogenous phosphatidylcholine were determined by studying the influence of pH, time and the concentrations of $CaCl_2$, sodium deoxycholate and phosphatidylcholine. The activity was optimal at pH 8.5, linear with protein concentration until at least 0.6 mg/ml and linear with incubation time for at least 80 min (not shown). The deacylation was dependent on the presence of Ca^{2+} (optimal concentration 10 mM, not shown). Other divalent cations were either totally ineffective or replaced Ca^{2+} to a very limited extent (Table 1). When using phosphatidylcholine as substrate, the activity was barely detectable in the absence of sodium deoxycholate (Fig. 1A). The optimal deoxycholate concentration depended on the concentration of substrate (not shown) and, in general, nearly equimolar amounts of bile salt and phosphatidylcholine were required. Concentrations of substrate around and below the critical micellar concentration of deoxycholate (20) required a proportionally higher amount of detergent, presumably reflecting the inability of the bile salt to stimulate hydrolysis at levels incompatible with micelle formation (note also the lag phase in Fig. 1B). Using optimal deoxycholate/phosphatidylcholine molar ratios, saturation kinetics could be obtained (not shown). However, the observation that deoxycholate precipitates with Ca^{2+} prompted us to look for a detergent compatible with that cofactor. Since the precipitation mentioned above and noted previously by others (21) is due to the free carboxylic group (22), a bile acid conjugated with an amino sulfonic acid was an obvious alternative to deoxycholate. Therefore, sodium taurodeoxycholate was chosen and its ability to stimulate phospholipase A_2 was compared with that of its nonconjugated counterpart. In addition, since the charge of substrate dispersions is known to be an important factor for the

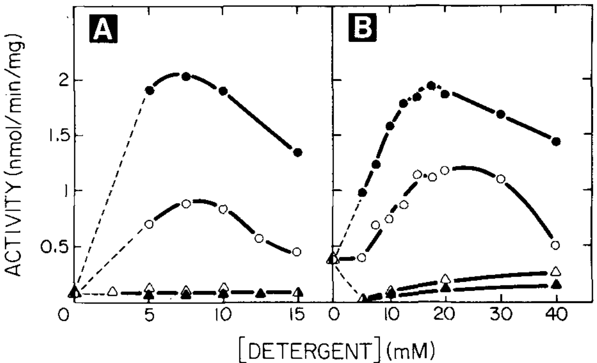

FIG. 1. Effects of different detergents on lung microsomal phospholipase A_2 activity. Except for the detergent used—sodium deoxycholate (○), sodium taurodeoxycholate (●), CHAPS (△) or Triton X-100 (▲)—at the indicated concentrations, standard incubation mixtures were carried out either with phosphatidylcholine (A) or phosphatidylethanolamine (B). For Triton X-100, an average M_r = 646 Da was used.

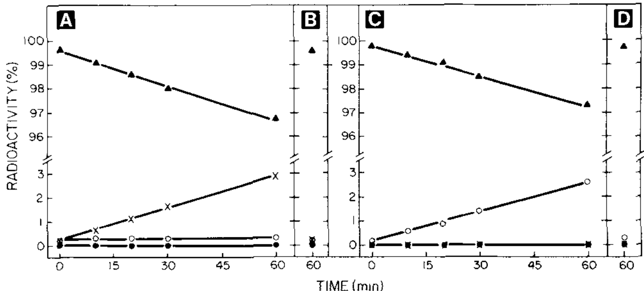

FIG. 2. Distribution of radioactivity after incubation of two differently labeled phosphatidylcholines with rabbit lung microsomes. Standard incubation mixtures were used except for the substrate, which was either 1-palmitoyl-2-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (A, B) or 1,2-dipalmitoyl-*sn*-glycero-3-phospho[*Me*-^{14}C] choline (C, D). Panels B and D represent blank incubations without microsomes. At the indicated times, the mixtures were adjusted to 1 ml with 19 mM H_2SO_4 and extracted (24). After evaporation under N_2, the chloroform-methanol phases were analyzed by thin-layer chromatography (25), and the areas corresponding to phosphatidylcholine (▲), lysophosphatidylcholine (○) and fatty acids (×) were scraped and counted for radioactivity. The water-methanol phases were evaporated overnight at 60 C and counted for radioactivity (●). Results are expressed as percentage of the total radioactivity recovered.

TABLE 1

Effect of Different Divalent Cations on Phospholipase A_2 Activity from Lung Microsomes

Cation	Activity (%)
Ca^{2+}	100
Cd^{2+}	10
Sr^{2+}	8
Mn^{2+}	7
Mg^{2+}	6
Ba^{2+}	4
Co^{2+}	2
Ni^{2+}	2
Cu^{2+}	2

Standard incubation conditions were used with phosphatidylcholine as substrate, except that divalent cations (as chlorides) were added to give final concentrations of 10 mM. Data are expressed as percentage of the activity obtained with $CaCl_2$ (1.8 nmol/min/mg) after correction for background hydrolysis in the absence of added ions (21 pmol/min/mg).

activity of a number of lipolytic enzymes (23), some incubations were performed with the zwitterionic bile salt analogue CHAPS or with the nonionic detergent Triton X-100. Parallel experiments were conducted with phosphatidylcholine (Fig. 1A) and phosphatidylethanolamine (Fig. 1B) as exogenous substrates. For ease of comparison, all the incubations depicted in Figure 1 were done with the same microsomal preparation. It is clear that in the absence of detergent, phosphatidylethanolamine was hydrolyzed at least sevenfold faster than phosphatidylcholine. In keeping with the results previously obtained with phosphatidylcholine (Fig. 1A), the addition of sodium deoxycholate increased the enzymatic specific activity toward phosphatidylethanolamine (Fig. 1B). However, the optimal bile salt concentration was considerably higher for this substrate (note the different scales for detergent concentration in panels A and B, Fig. 1). Regarding the stimulation of activity toward phosphatidylcholine and phosphatidylethanolamine, it is interesting to note that the addition of sodium deoxycholate to mixtures of $CaCl_2$ and substrate produced macroscopic changes that were much more dramatic in the case of phosphatidylethanolamine. This occurred even at the lower detergent levels (5-15 mM) and resulted in the separation of large aggregates (presumably containing phosphatidylethanolamine, Ca^{2+} and deoxycholate) from an optically clear liquid phase.

Optimal concentrations of taurodeoxycholate stimulated the activity to about twice the levels obtained with optimal concentrations of deoxycholate (Fig. 1). Again, phosphatidylethanolamine required relatively higher detergent concentrations.

On the other hand, addition of either CHAPS or Triton X-100 failed to change the very low activities obtained with exogenous phosphatidylcholine in the absence of any detergent (Fig. 1A), and both detergents inhibited the deacylation of phosphatidylethanolamine, particularly at concentrations of 5-10 mM (Fig. 1B).

The specificity of the deacylating activity regarding the two acyl chains of the substrate was investigated by carrying out two similar incubations, one using phosphatidylcholine radiolabeled in the *sn*-2 acyl chain and the other using a choline-labeled substrate, and analyzing the distribution of radioactivity at various time intervals. The specificity of the microsomal phospholipase toward the *sn*-2 acyl chain of the substrate can be concluded from the absence of any measurable phospholipase A_1 (Fig. 2A) and any phospholipase B (or a combination of a phospholipase A_1 and a lysophospholipase) (Fig. 2C). These findings were corroborated in a more quantitative manner by the stoichiometry of the reaction: calculations based on the slopes of the regression lines depicted in Figure 2A showed that palmitate became unesterified at a rate comparable to that of phosphatidylcholine hydrolysis (0.76 and 0.72 nmol/min, respectively). Similarly, for the choline-labeled substrate (Fig. 2C), the rate of phosphatidylcholine degradation equaled that of lysophosphatidylcholine formation (0.66 nmol/min).

Since at some stage in its purification the microsomal phospholipase A_2 will likely require the use of salt, we investigated the effect of KCl on the activity. Again, phosphatidylcholine (Fig. 3A) or phosphatidylethanolamine (Fig. 3B) were used as substrates and optimal concentrations of deoxycholate or taurodeoxycholate were employed in each case (cf. Fig. 1). Phosphatidylethanolamine was also used in the absence of any detergent. As shown in Figure 3, the KCl effects were qualitatively independent of the substrate used but strongly dependent on whether the assay system contained deoxycholate (activation) or taurodeoxycholate (inhibition).

The influence of several compounds on lung microsomal phospholipase A_2 activity is indicated in Table 2. In keeping with the Ca^{2+} dependency mentioned

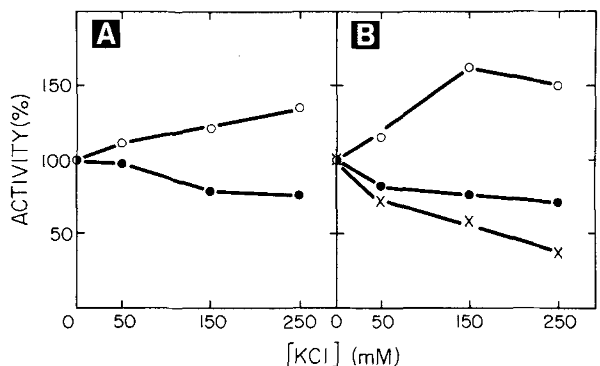

FIG. 3. Effects of KCl on lung microsomal phospholipase A_2 activity. The enzymatic activities were determined in the presence of the indicated KCl concentrations with standard incubation mixtures containing phosphatidylcholine (A) and 7.5 mM sodium deoxycholate (○) or 7.5 mM sodium taurodeoxycholate (●); or phosphatidylethanolamine (B) and 17.5 mM sodium deoxycholate (○), 17.5 mM sodium taurodeoxycholate (●) or no detergent (×). Phospholipase A_2 activity is expressed as percentage of the activity obtained for each detergent/phospholipid combination without KCl.

TABLE 2

Influence of Various Reagents on Phospholipase A_2 Activity from Lung Microsomes

Reagent in preincubation	Concentration during preincubation (mM)	Residual activity (%)
None	—	100
EDTA	40	3
p-Bromophenacyl bromide	0.4	8
	4	2
N-Ethylmaleimide	8	65
Iodoacetamide	8	84
p-Hydroxymercuribenzoate	8×10^{-3}	97
	8	82
NaF	8	95
Diisopropylfluorophosphate	8	90

Microsomes (80 μg protein) were preincubated at 37 C in the presence of the indicated reagents (total volume 123 μl). After 30 min, the tubes were placed in ice, and phosphatidylcholine, EDTA and taurodeoxycholate were added to give standard assay concentrations (see Materials and Methods). The assay of residual phospholipase A_2 activity was initiated by addition of $CaCl_2$ (final concentration 10 mM) and incubation at 37 C (total volume 0.2 ml). Results are expressed as percent activity relative to that of microsomes preincubated with buffer alone (3 nmol/min/mg).

above, an excess of EDTA resulted in an almost complete loss of activity. While virtually no activity could be recovered after preincubation of microsomes with the alkylating reagent *p*-bromophenacyl bromide, no marked inhibitory effects were obtained with diisopropylfluorophosphate or fluoride. Among the thiol reagents tested, only N-ethylmaleimide reduced the activity to a considerable extent.

DISCUSSION

The optimal pH and Ca^{2+} levels required by phospholipase A_2 from rabbit lung microsomes are similar to those reported for the microsomal enzyme from rat lung assayed with either exogenous (10) or endogenous (12) substrates. However the results obtained with divalent cations (Table 1) contrast with reports indicating that some of these ions, particularly Sr^{2+} and Mg^{2+}, can partially support other Ca^{2+}-dependent phospholipases (26–28).

In agreement with previous evidence obtained from rat lung microsomes (12), microsomal preparations obtained from rabbit lung also failed to significantly deacylate exogenous phosphatidylcholine (Fig. 1A). However, hydrolysis could be greatly stimulated by the addition of sodium deoxycholate (Fig. 1A and Ref. 17). In a search for a Ca^{2+}-compatible detergent, we found that replacement of deoxycholate with taurodeoxycholate resulted in a roughly twofold stimulation of the activity (Fig. 1). Interestingly, the optimal Ca^{2+} concentration did not change when taurodeoxycholate replaced deoxycholate (not shown). The importance of the detergent's negative charge in bile salt stimulation of the microsomal phospholipase is suggested by the failure of CHAPS to stimulate the activity (Fig. 1). This detergent is a zwitterionic bile salt analogue whose resemblance to bile salts extends to its membrane-solubilizing properties (29). Therefore, regarding its preference for negatively charged substrate-water interfaces, this enzyme is more similar to pancreatic than to snake venom phospholipases A_2 (30–33). This view is further strengthened by the experiments depicted in Figure 1 with Triton X-100, a nonionic detergent commonly employed in the assay of snake venom phospholipases A_2 (34).

The apparent preference of the pulmonary microsomal phospholipase for phosphatidylethanolamine over phosphatidylcholine in the absence of detergents (Fig. 1) has also been observed with a number of other membrane-bound phospholipases (35 and references therein). As recently shown for rat liver mitochondria, this preference does not reflect true enzyme specificity but rather the ability of phosphatidylethanolamine to achieve a more extensive association with the enzyme-containing membranes (35). As a corollary, it follows that there should be no difference in the rates of deacylation of these substrates when a preassociation of the substrate with enzyme-containing membranes is prevented, for example, by solubilization. Comparable enzymatic activities toward both substrates can indeed be obtained at optimal bile salt concentrations (Fig. 1), indicating that an interpretation similar to that derived for mitochondrial phospholipase (35) might be appropriate for the apparent specificity of the lung microsomal enzyme in the absence of detergents.

The experiments conducted with KCl (Fig. 3) illustrate how the addition of simple ions can cause opposite effects on phospholipase A_2 activity, simply by replacing a conjugated bile salt with its unconjugated counterpart. These results emphasize the need for caution when drawing general conclusions from lipolytic reactions conducted in the presence of detergents (2).

The data obtained with *p*-bromophenacyl bromide and with diisopropylfluorophosphate (Table 2) suggest the importance of a histidine rather than a serine

residue at the active site of the enzyme from lung microsomes and make it comparable, in this regard, to most of the phospholipases A_2 studied so far (1). In agreement with the view that this class of enzymes lacks free sulfhydryl groups (1), most of the thiol reagents tested were unable to appreciably inhibit the activity (Table 2). The reason why N-ethylmaleimide partially inhibited the enzyme is not clear at present but, in view of the fact that *p*-bromophenacyl bromide can react with sulfhydryl groups (36,37), the possibility remains that free sulfhydryls are critical for this enzyme's activity. Obviously, the clarification of this and other questions must await the eventual purification of the enzyme.

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Interferon α/β Induces Changes in the Metabolism of Polyenoic Phospholipids and Diacylglycerols in the Livers of Suckling Mice

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Suckling mice were injected daily from birth for 10 days with potent preparations of mouse interferon α/β. Interferon treatment resulted in a markedly lower concentration of polyunsaturated fatty acids (20:4ω6 and 22:6ω3) in the two principal liver phospholipids, phosphatidylcholine and phosphatidylethanolamine, than in livers of control-treated mice. This effect appeared to correlate with a low level of synthesis of polyunsaturated phospholipids in the livers of interferon-treated mice. Thus, in control mice, synthesis of species of polyunsaturated phospholipids increased markedly in the first 10 days of life, whereas in 10-day-old interferon-treated mice, the level of synthesis of species of polyunsaturated phospholipids was comparable to that in newborn mice. In parallel, a marked increase in the diacylglycerol content without change of its renewal was observed in the livers of interferon-treated mice. We suggest that interferon treatment results in an inhibition of one of the processes that leads to activation of the enzymatic systems responsible for the synthesis of species of polyunsaturated phosphatidylcholine and phosphatidylethanolamine in the liver of suckling mice. It seems likely that these results are related to the inhibition of liver cell maturation and the marked cell necrosis that are observed in interferon-treated suckling mice.
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Administration of potent preparations of interferon α/β to suckling mice in the first week of life results in inhibition of growth, diffuse liver cell necrosis and death (1). Recently we showed that this interferon treatment resulted in a marked increase in liver triglycerides and a decrease in the level of some hepatic phospholipids (2). Whereas the percentage of liver phospholipids (PL) (relative to liver protein) increased in the first week of life for normal suckling mice, it decreased in the livers of interferon-treated mice (2). These biochemical modifications, not observed in adult mice (2), were accompanied by the appearance of abnormal tubular aggregates arising from the endoplasmic reticulum of hepatocytes (3,4), suggesting important alterations in the lipid-protein interactions at the level of these membranes (5).

There have been several reports indicating that, in vitro, interferon induced changes in the fatty acid composition of PL in cells. Thus, Chandrabose et al. (6) observed that mouse interferon induced a decrease in the unsaturated fatty acid content of all the major PL in mouse sarcoma cells, and Apostolov and Barker (7,8) showed that human interferon reduced the C18 unsaturated fatty acid content of human fibroblasts and bovine kidney cells. These changes may contribute to the observed increased rigidity of the plasma membrane lipid bilayer of some interferon-treated cells (9). The results presented herein indicate that in vivo, interferon induces similar biochemical modifications in suckling mice as a result of alterations in the metabolism of the polyunsaturated molecular species of liver PL.

MATERIAL AND METHODS

Mice. Litters of Swiss mice were obtained from the pathogen-free colony at the Institut Recherches Scientifiques sur le Cancer, Villejuif, France.

Interferon preparation. Mouse interferon α/β was prepared from suspension cultures of mouse sarcoma C243 cells inoculated with Newcastle disease virus (NDV). The methods of production and purification have previously been described (10). Interferon was assayed by inhibition of cytopathic effect of vesicular stomatitis virus on L cells in monolayer culture (0.2 ml/well) in Falcon microplates. Units in the text are expressed as international mouse reference units. The specific activity of this purified interferon was ca. 2×10^7 units/mg protein. Control preparations consisted of supernatant from cultures of C243 cells prepared in exactly the same way as interferon except that the interferon inducer, NDV, was omitted.

Experimental plan. Half of the mice in each litter were marked and injected subcutaneously in the intercapsular region daily from birth to the sixth or tenth day with 0.05 ml of purified mouse interferon having a titer of 3.2×10^6/ml or with the control preparation. Mice were killed on the sixth or the tenth day. After mice were killed , the livers were weighed; a piece was removed for histologic examination and the remaining tissue was weighed, ground with a blender in 10 ml of chloroform/methanol (1:1,v/v) and stored at -80 C. The lipid extraction was performed according to Folch et al. (11).

Separation of neutral lipids and total PL for analysis of fatty acids. Lipids were chromatographed on a silica gel (Mallinckrodt, Paris KY) column. After the column was washed with hexane/isopropyl ether (90:10, v/v), triacylglycerols were eluted by hexane/isopropyl ether (70:30, v/v), diacylglycerois and all nonphosphorus-containing lipids by isopropyl ether/methanol (95:5, v/v) and PL by methanol (93-95% recovery). Aliquots of triacylglycerol and 1,2-diacylglycerol fractions were purified by thin layer chromatography (TLC) on silica gel G 60 precoated plates (Merck, Darmstadt, FRG) in heptane/isopropyl ether acetic acid (60:40:4 v/v/v) solvent system (12). The 1,2-diacylglycerol and triacylglycerol spots, detected under UV light after spraying with primuline reagent

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Abbreviations: PL, phospholipid; NDV, Newcastle disease virus; TLC, thin layer chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; DG, diacylglycerol; TG, triacylglycerol.

(13), were scraped into screw-capped tubes. A known amount of 17:0 fatty acid was added as a tracer to each fraction, and transesterification by methanolic-HCl was performed as previously described (14).

Fatty methyl esters were analyzed using a gas liquid chromatograph (Packard 428, Downers Grove, IL) with a 10% SP 2330 on 100/120 chromosorb WAW column of 9′ 1/16″ SS operated isothermally at 190 C. Results are expressed as percent of total fatty acids by means of a coupled integrator (Packard instrument).

The amount of fatty acid in each of the samples was calculated by comparing the surface of each of the peaks to the surface of the peak of 17:0 on the same chromatogram.

Analysis of fatty acids in phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI). PL from the fraction previously purified by column were separated by TLC using chloroform/methanol/acetic acid/water (60:30:7:3, v/v/v/v) as developing solvent (15). PC, PE, PS and PI spots visualized under UV light after spraying with primuline were scraped into screw-capped tubes, and methyl esters of fatty acids were prepared as described above.

In vivo measurement of PL biosynthesis in the liver of suckling mice. Suckling mice were killed 5,20,45 or 60 min after injection of 150 μCi of [2-^{3}H] glycerol per 5 g of mouse. Livers were homogenized in 20 vol of chloroform/methanol (1:1, v/v) and the protein precipitate, separated from the organic phase by centrifugation, was reextracted by two other extractions with this solvent mixture. The pooled organic phases were adjusted to 2:1 chloroform/methanol and subsequently washed as previously described (14,16). Total lipid phosphorus and lipid radioactivity was determined on aliquots of lipids extracted from liver.

Neutral lipids were resolved by TLC on silica gel G 60 precoated plates in monodimension with heptane/isopropyl ether/acetic acid (60:40:4, v/v/v). Lipids were detected under UV light with primuline reagent, or standards were chromatographed in parallel and stained with vanillin/sulfuric acid spray reagent.

PL were separated by two-dimensional TLC on 10 × 10 cm silica gel precoated plates using solvents previously described (17). The spots on the chromatogram were visualized with the phosphorus-specific spray reagent of Dittmer and Lester (18).

Visualized spots were scraped into a counting vial, and 3 ml of ethanol/water (1:1, v/v) and 8 ml of Picofluor-30 (Packard) were successively added (19). After mixing, each vial was counted in a Tricarb spectrometer (Packard). The counting efficiency was determined by the external-standard channel ratio, and the results are expressed in dpm.

Proteins in the delipidized precipitate were determined by the method of Lowry et al. (20) after dissolving in 1 N NaOH.

Triacylglycerols were determined by enzymatic method (21) on aliquots of total lipid and triacylglycerol chromatographic fraction dissolved in a minimum volume of propanol. The proportions of 1,2-diacylglycerols in neutral lipids were determined in the corresponding fraction of column chromatography by the same enzymatic method after separation of

TABLE 1.

Effect of Interferon on the Composition of Fatty Acids in Liver Phospholipids of Suckling Mice[a]

	Age of mice				
		6 Days		10 Days	
Fatty acids	At birth (8)[b]	Control (4)	IFN-treated (4)	Control (5)	IFN-treated (6)
16:0	25.0 ± 0.3	21.2 ± 0.3	21.7 ± 0.1	26.1 ± 0.5	26.8 ± 0.3
16:1	1.3 ± 0.1	0.9 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.9 ± 0.1
16:2	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	1.0 ± 0.1
18:0	16.6 ± 0.3	17.3 ± 0.1	17.6 ± 0.7	16.3 ± 0.2	16.6 ± 0.1
18:1	11.5 ± 0.1	8.9 ± 0.4	12.1 ± 0.4	7.5 ± 0.1	10.7 ± 0.3
18:2ω6	10.4 ± 0.1	10.3 ± 0.2	10.2 ± 0.1	10.6 ± 0.2	11.4 ± 0.2
18:3ω6	0.7 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
18:3ω3	1.1 ± 0.1	1.2 ± 0.5	1.4 ± 0.1	0.5 ± 0.1	1.0 ± 0.2
20:3	1.3 ± 0.1	1.9 ± 0.1	2.5 ± 0.1	1.7 ± 0.2	1.2 ± 0.2
20:4ω6	12.2 ± 0.4	15.7 ± 0.2	14.2 ± 0.4	14.1 ± 0.2	10.4 ± 0.2
20:5ω3	2.5 ± 0.1	2.4 ± 0.4	2.3 ± 0.1	1.7 ± 0.1	1.6 ± 0.1
22:3	0.3 ± 0.1	0.9 ± 0.4	1.0 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
22:5ω3	0.6 ± 0.1	0.9 ± 0.1	1.7 ± 0.1	1.0 ± 0.1	1.7 ± 0.1
22:6ω3	14.8 ± 0.5	15.3 ± 0.8	12.0 ± 0.8	17.1 ± 0.5	14.6 ± 0.4
Σω6	23.3	26.5	25.2	25.1	22.2
Σω3	18.9	19.8	18.5	20.3	18.9
U.I.[c]	198	215	193	212	191

[a]The results expressed as the percentage of total fatty acids are means ± SM.
[b]Number in parentheses indicates the number of mice. Newborn and 10-day-old mice came from the same litters, whereas 6-day-old mice came from a second experiment at a different time of year.
[c]U.I., the unsaturation index, is the sum of the percentage multiplied by the number of double bonds for each fatty acid in the mixture.

the 1,2-diacylglycerols in parallel with a known amount of purified diacylglycerols by TLC as described above. The amount of diacylglycerols on the plate was calculated from the corrected OD obtained with the standard spot of diacylglycerols on the same plate.

Separation of molecular species of radioactive PC and PE. PC and PE were purified from the PL fraction by preparative TLC on 20 × 20 cm plates coated with silica gel N, HR (Macheray-Nagel, Düren, FRG) containing 1% sodium carbonate in chloroform/methanol/acetic acid/water (60:30:7:3, v/v/v/v) (15). PC and PE, localized by fluorescence after plates were sprayed with primuline, were eluted from gel by extraction with a mixture of chloroform/methanol/ acetic acid/ water (50:39:1:10, v/v/v/v) (22). The different molecular species of PC and PE were separated by chromatography on 5 × 10 cm silica gel G 60 precoated plates impregnated with silver nitrate (23,24). In brief, the plates were immersed for 30 min in a solution of 50 g of silver nitrate dissolved in 50 ml of water, then dried for 10 min at 110 C and activated for 8 min at 170 C before use. Each purified PL (about 2 mg) was spotted on each plate, and chromatography was performed in a chloroform/methanol/ water solvent system (60: 40:7.5. v/v/v) for PC and (70:40:10, v/v/v) for PE. After drying, the four major separated fractions were identified by the reagent of Dittmer and Lester, scraped into counting vials and counted as described above.

RESULTS

Effect of interferon on the fatty acid composition of phospholipids in the livers of suckling mice (Table 1). Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2ω6), eicosatetraenoic (20:4ω6) and docosahexaenoic (22:6ω3) acids are the major fatty acids in the liver PL of suckling mice (Table 1). In the first 10 days of life, the proportion of polyunsaturated fatty acids in liver PL increased (43.7% at birth to 47.5% for 10-day-old mice). This overall increase in polyunsaturated acids in PL was not observed in interferon-treated suckling mice. Thus, the percentage of 20:4ω6 and 22:6ω3 in PL increased by 15% for control mice (compared to newborn mice) and either decreased for interferon-treated mice at 10 days or showed the same value as for newborn mice. The percentage of oleic acid declined with age for control mice, but for interferon-treated mice it stayed at the same level as for newborn mice. It is interesting to note that the polyunsaturated fatty acid index of interferon-treated 10-day-old mice was closer to that of newborn mice than it was to control 10-day-old suckling mice.

Effect of interferon on the fatty acid composition of the major liver PL of suckling mice (Table 2). In PC, there was a clear-cut decrease in the percentage of eicosatetraenoic (20:4ω6) and docosahexaenoic (22:6ω3) acids and an increase in the percentage of palmitoleic (16:1), oleic (18:1), linoleic (18:2ω6) and eicosapentaenoic (20:5ω3) acids in interferon-treated

TABLE 2

Effect of Interferon on the Fatty Acid Composition of Phosphatidylcholine (PC), Phosphatidylethanolamine (PE) and Phosphatidylserine + Phosphatidylinositol (PS + PI) in Liver of 10-Day-Old Mice[a]

	PC		PE		PS+PI	
Fatty acids	Control	IFN-treated	Control	IFN-treated	Control	IFN-treated
16:0	34.5 ± 0.9	35.2 ± 0.8	22.8 ± 0.3	19.9 ± 0.4	14.9 ± 0.1	15.2 ± 0.2
16:1	0.9 ± 0.1	1.2 ± 0.1	ND	0.6 ± 0.1	ND	ND
18:0	13.0 ± 0.2	13.8 ± 0.2	22.1 ± 0.2	21.6 ± 0.2	39.6 ± 0.7	37.0 ± 0.6
18:1	8.7 ± 0.1	12.8 ± 0.1	4.3 ± 0.1	8.7 ± 0.1	5.5 ± 0.2	7.3 ± 0.2
18:2ω6	12.7 ± 0.2	13.5 ± 0.1	2.8 ± 0.1	8.5 ± 0.1	2.1 ± 0.1	3.4 ± 0.1
18:3ω6	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	ND	ND	ND
18:3ω3	0.8 ± 0.1	1.4 ± 0.1	0.7 ± 0.1	1.3 ± 0.1	0.3 ± 0.1	1.0 ± 0.6
20:3ω6	2.3 ± 0.1	1.9 ± 0.3	1.1 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	1.6 ± 0.6
20:4ω6	10.8 ± 0.1	8.1 ± 0.4	17.1 ± 0.3	13.8 ± 0.3	21.7 ± 0.1	18.7 ± 0.3
20:5ω3	1.0 ± 0.1	1.5 ± 0.2	2.1 ± 0.2	2.7 ± 0.2	1.0 ± 0.1	1.3 ± 0.1
22.5ω3	0.6 ± 0.1	0.9 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	0.8 ± 0.1	1.6 ± 0.1
22:6ω3	13.3 ± 0.2	8.1 ± 0.1	24.3 ± 0.1	19.5 ± 0.2	12.0 ± 0.1	11.6 ± 0.5
Σω6	26.2	24.0	21.7	23.4	24.5	23.6
Σω3	15.8	11.8	28.5	25.1	14.1	15.6
ω6/ω3	1.7	2.0	0.8	1.0	1.7	1.5
U.I.[b]	176	144	248	227	181	181

[a]The results are given in percentage of total fatty acids recovered and are expressed as means ± SE. Four pools of two livers were analyzed: each determination was done in duplicate. Minor fatty acids as 12:0, 14:0, 14:1, 15:0, 17:0, 20:0, 20:1, 22:1 and 24:0 are not included in the table, but for the determination of total fatty acids all identified acids were included.
[b]U.I., the unsaturation index, is the sum of the percentage multiplied by the number of double bonds for each fatty acid in the mixture.
ND, Not detected.

mice. The unsaturation index decreased from 176 for control mice to 144 for interferon-treated mice.

PE contains the highest percentage of unsaturated fatty acids of liver PL. Interferon treatment resulted in a decrease in palmitoleic, eiocosatetraenoic and docosahexaenoic acids, accompanied by an increase in the percent of oleic, linoleic and linolenic in ω3 (18:3ω3) fatty acids. The overall unsaturation index decreased from 248 for control mice to 227 for interferon-treated mice.

No change in the unsaturation index was observed in the fractions PS and PI.

Effect of interferon on the amount of fatty acids in the livers of suckling mice. The results presented above give only an imperfect picture of the extent of the biochemical changes due to interferon treatment, as in the first 10 days of life the liver weight increases from 83 mg at birth to 208 mg (10 days) for control mice and 260 mg for interferon-treated mice, and the amount of liver lipids increases from 3.1 mg/liver at birth to 9.8 mg for control mice and 25 mg for interferon-treated mice (2). For this reason, we have quantitated fatty acids in the liver PL using an internal standard of 17:0. Thus, per liver, the total amount of palmitic acid (16:0) doubled, and oleic (18:1) and linoleic (18:2ω6) acids tripled or quadrupled in interferon-treated mice (Table 3). However, in the two groups of mice, the levels of polyunsaturated fatty acids in C20 and C22 were virtually equal; only the sum of unsaturated fatty acids in ω6 was increased in interferon-treated mice as a result of the marked increase in linoleic acid (18:2ω6).

In contrast, there was a marked decrease in the amount per liver of fatty acid present in the liver PL (i.e., 8.8μmol per liver for control mice to 5.2 μmol for interferon-treated mice). Palmitoleic (16:1), oleic (18:1), linolenic (18:3ω6 and 18:3ω3) and docosatrienoic (22:3) acids present in PL decreased ca. 20%; palmitic (16:0), stearic (18:0), eicosatrienoic (20:3) and eicosapentaenoic (20:5) acids decreased 40%; eicosatetraenoic (20:4ω6) and docosahexaenoic (22:6ω3) decreased more than 50%. Only docosapentaenoic acid (22:5ω3) increased by 8%.

It can be seen, therefore, that the polyunsaturated fatty acids in PL were the most affected by interferon treatment. (For control mice, polyunsaturated fatty acids in phospholipids represent 4.3 μmol per liver, whereas they only constitute 2.3 μmol for interferon-treated mice).

Effect of interferon on biosynthesis of lipids in the livers of suckling mice: kinetics of incorporation of 1(3)-^{3}H glycerol. Five min after injection of radioactive glycerol, 50% of the lipid radioactivity was present in triacylglycerides. The level of radioactivity in triacylglycerides then decreased for 8-day-old control mice, whereas it increased for interferon-treated mice (Fig. 1). The radioactivity in diacylglycerides was greater in interferon-treated mice than in control mice and decreased in parallel with time in both groups (Fig. 1). The radioactivity in PL increased in parallel for both groups, although the amount in interferon-treated mice was about half that of control mice (Fig. 1). Although there was a marked decrease in the PL content of the liver in interferon-treated suckling mice (2.1

TABLE 3

Effect of Interferon on the Amount of Fatty Acids in Total Lipids and Phospholipids of the Livers of 10-Day-Old Suckling Mice[a]

	Total lipids		Phospholipids	
Fatty Acids	Control (6)	IFN-treated (6)	Control (6)	IFN-treated (6)
16:0	3.1 ± 0.2	6.1 ± 0.5**	2.14 ± 0.12	1.32 ± 0.14**
16:1	0.2 ± 0.1	0.8 ± 0.2	0.07 ± 0.01	0.05 ± 0.01**
18:0	1.7 ± 0.2	1.8 ± 0.3	1.51 ± 0.02	0.91 ± 0.06**
18:1	2.5 ± 0.4	8.6 ± 1.2**	0.73 ± 0.01	0.60 ± 0.03*
18:2ω6	1.5 ± 0.2	3.7 ± 0.4**	0.94 ± 0.03	0.58 ± 0.05**
18:3ω6	0.1 ± 0.1	0.2 ± 0.1	0.04 ± 0.01	0.03 ± 0.01
18:3ω3	ND	ND	0.08 ± 0.01	0.06 ± 0.01
20:3	0.2 ± 0.1	0.5 ± 0.1	0.16 ± 0.01	0.09 ± 0.01**
20:4ω6	1.4 ± 0.1	1.1 ± 0.2	1.33 ± 0.01	0.63 ± 0.02**
20:5ω3	0.2 ± 0.1	0.5 ± 0.2	0.18 ± 0.01	0.10 ± 0.01**
22:3	ND	ND	0.06 ± 0.01	0.05 ± 0.01
22:5ω3	0.1 ± 0.1	0.4 ± 0.1	0.08 ± 0.01	0.09 ± 0.01
22:6ω3	1.5 ± 0.3	1.2 ± 0.2	1.46 ± 0.06	0.71 ± 0.08**
Σω6	3.2 ± 0.3	5.3 ± 0.5**	2.31 ± 0.02	1.24 ± 0.03**
Σω3	2.0 ± 0.2	2.7 ± 0.4	1.80 ± 0.04	0.96 ± 0.05**

[a]The amount in μmol/liver of each fatty acid is mean± SM and is determined by comparison with an internal standard of 17:0 fatty acid introduced in the lipids at the time of transesterification (see Materials and Methods). Calculations take into account the surface of the peaks of fatty acids with that of standard on the same chromatogram. Number in parentheses indicates the number of mice. Values statistically different between control and IFN-treated:*, $p < 0.05$; **, $p < 0.01$.
ND, not detected.

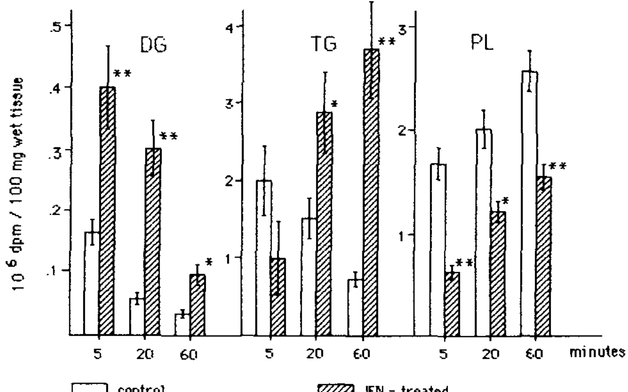

FIG. 1. Effect of interferon treatment on the kinetics of 1(3)-^{3}H-glycerol incorporation into the diacylglycerols (DG), triacylglycerols (TG) and phospholipids (PL) of the livers of suckling mice. Each value was the mean ± SM of dpm/100 mg of wet tissue. Values statistically different between control and IFN-treated; *, $p < 0.05$; **, $p < 0.01$.

± 0.2% of wet tissue compared with 3.6 ± 0.3% in control mice), the specific radioactivity of PL was comparable between the 2 groups and increased regularly between 5 and 60 min (for control mice, 450 ± 60 dpm/μg of PL [5 min] to 560 ± 40 dpm/μg [20 min] and 710 ± 50 [60 min], whereas for interferon-treated mice, the values were 290 ± 80 [5 min], 570 ± 50 [20 min] and 740 ± 40 [60 min]). There was no significant difference in the distribution of radioactivity in the different species of PL.

Comparison of PL synthesis in the livers of newborn and 10-day-old control and interferon-treated mice. Incorporation of ^{3}H-glycerol in liver PL was determined 45 min after injection of the tracer. As can be seen in Table 4, the amount of radioactivity per 100 mg liver protein doubles or triples in control mice between birth and 10 days, whereas the levels in interferon-treated mice are comparable to those found in newborn mice.

Distribution of radiolabel among the different molecular species of PC and PE (Table 5). For 10-day-old control mice, the radiolabel in PC was found almost equally distributed in the low unsaturated (i.e., monoenoic and dienoic) and polyunsaturated (i.e., tetraenoic and hexaenoic) species. In contrast, in 10-day-old interferon-treated mice, only 37% of the radioactivity was found in the polyunsaturated species, as was also found in newborn mice.

For PE, in 10-day-old control mice, the major part (82%) of the radioactivity was found in the polyunsaturated species. Interferon treatment resulted in a decrease in this percentage to 66%, which was similar to the percent of radioactivity in newborn mice.

In summary, interferon treatment affected preferentially the synthesis of molecular species of PC and PE containing mostly polyunsaturated fatty acids.

Effect of interferon on the composition and metabolism of 1,2-diacylglycerols and triacylglycerols. As interferon treatment of suckling mice resulted in an abnormal accumulation of 1(3)-^{3}H-glycerol in 1,2-diacylglycerols and as these compounds are common intermediates for triacylglycerol and phospholipid biosynthesis, we determined the concentration and the specific radioactivity of 1,2-diacylglycerols and their fatty acid composition. Interferon treatment increased the concentration of 1,2-diacylglycerols about four- to fivefold in the liver (from 0.4 ± 0.3 μmol/g of wet tissue in control mice to 2.1 ± 1.0 μmol/g in interferon-treated mice), although their average specific radioactivity remained almost equivalent (1787 ± 291 dpm/μg of diacylglycerols for control mice and 1591 ± 230 dpm/μg for interferon-treated mice). Moreover, there was only a significant decrease of 18:1

TABLE 4

Radioactivity Incorporated in the Liver Phospholipids of Newborn Mice and 10-Day-Old Control and Interferon-Treated Mice*

		10-Day-old mice	
	Newborn (8)	Control (6)	IFN-treated (6)
LPC	1,013 ± 150[a]	2,112 ± 276	563 ± 172[a]
SPH	454 ± 55[b]	616 ± 114[b]	318 ± 19[b]
PC	110,160 ± 6,082[c]	263,976 ± 22,220	105,268 ± 20,912[c]
PS	1,201 ± 130[d]	2,219 ± 258[e]	1,211 ± 244[e,d]
PI	6,738 ± 403[f]	15,532 ± 1,830	5,769 ± 1,096[f]
PE	62,803 ± 3,827[g]	169,406 ± 14,356	56,746 ± 11,879[g]
DPG	2,768 ± 210[h]	2,147 ± 215[h]	1,611 ± 309[h]
PA	613 ± 51[i]	1,198 ± 134[j]	976 ± 207[i,j]
TG	160,066 ± 21,735[k]	231,116 ± 20,358[k]	294,982 ± 59,655[k]

*Values are means ± SE of dpm/100 mg of liver proteins. The radioactivity in each phospholipid was measured 45 min after injection of 150 μCi of 1(3)-^{3}H-glycerol/5 g of mouse. Each chromatogram of labeled lipids was done in duplicate and the radioactivity was determined in each of the chromatograms. Number of parentheses indicates the number of mice per group. Values with a common superscript are not significantly different. LPC, lysophosphatidylcholine; PA, phosphatidic acid; SPH, sphingomyelin; DPG, diphosphatidylglycerol; TG, triacylglycerols.

(from 32.5 to 26.3%) and a nonsignificant increase of 20:4 and 22:6 in the diacylglycerols (DG) of interferon-treated mice (Table 6).

These variations cannot explain, therefore, the interferon-induced decrease in the proportion of polyunsaturated fatty acids in liver PL. The same arguments hold for the triacylglycerols, whose concentration was increased five- to sixfold by interferon treatment (2) and where only a significant increase of 18:1 was observed in the fatty acid composition between the two groups (Table 6).

DISCUSSION

The experimental results presented herein emphasize the very marked effects of interferon on lipid metabolism in the livers of suckling mice. It seems, therefore, that the interferon-induced alterations at the level of neutral lipids and PL do not have the same origin. Thus, we have previously observed, on one hand, a retention of newly formed triacylglycerols in the liver without any modification of their fatty acid composition and, on the other hand, a decrease in the total

TABLE 5

Distribution of the 1(3)-^{3}H-Glycerol Among Molecular Species of Liver Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) in Newborn Mice and 10-Day-Old Control and Interferon-Treated Mice[a]

	PC			PE		
		10-Day-old			10-Day-old	
Molecular Species[b]	Newborn	Control (6)[c]	IFN-treated (6)	Newborn	Control (6)	IFN-treated (6)
Monoenoic	30	18 ± 1	28 ± 3*	17	8 ± 1	16 ± 2*
Dienoic	32	31 ± 4	35 ± 3	16	10 ± 1	19 ± 2*
Tetraenoic	21	23 ± 3	19 ± 2	31	35 ± 4	29 ± 3
Hexaenoic	17	27 ± 4	18 ± 2	38	47 ± 5	37 ± 4

[a]Results are expressed as dpm percentage of the total dpm recovered from the four fractions separated on the chromatogram.
[b]Each molecular species is defined by its most unsaturated fatty acid.
[c]Number in parentheses indicates number of determinations. The distribution of 1(3)-^{3}H-glycerol among PC and PE of different degrees of saturation at each time-interval was analyzed by argentation TLC. In 10-day-old suckling mice, the distribution of radiolabel did not change with time during the experimental period, and the results were the means ± SM of analyses 5, 20 and 60 min after injection of labeled glycerol (six pools of lipids from two animals). At birth, PC and PE were analyzed 45 min after label injection (two pools of lipids from three animals each).
*Value statistically different between control and IFN-treated, $p < 0.01$.

TABLE 6

Effect of Interferon on the Fatty Acid Composition of 1,2-Diacylglycerol and Triacylglycerol in the Livers of 10-Day-Old Suckling Mice[a]

	1,2-Diacylglycerol		Triacylglycerol	
Fatty acids	Control (3)[b]	IFN-treated (3)	Control (6)	IFN-treated (6)
16:0	26.4 ± 0.2	27.2 ± 0.5	23.3 ± 0.6	24.7 ± 0.5
16:1	2.0 ± 0.1	3.1 ± 0.1	2.6 ± 0.1	2.9 ± 0.1
18:0	5.0 ± 0.1	4.8 ± 0.1	5.3 ± 0.1	4.3 ± 0.1
18:1	32.5 ± 0.7	26.3 ± 0.7	27.6 ± 1.1	33.6 ± 0.3
18:2ω6	15.4 ± 0.3	14.2 ± 0.5	15.4 ± 0.5	15.3 ± 0.2
18:3ω6	ND	ND	1.1 ± 0.1	0.6 ± 0.1
18:3ω3	3.7 ± 0.1	2.8 ± 0.2	4.6 ± 0.2	4.7 ± 0.2
18:4	ND	ND	1.1 ± 0.1	0.6 ± 0.1
20:3	1.1 ± 0.6	1.2 ± 0.1	0.6 ± 0.3	1.1 ± 0.1
20:4ω6	7.8 ± 0.9	10.7 ± 0.8	1.8 ± 0.3	1.4 ± 0.1
20:5ω3	ND	ND	1.1 ± 0.3	0.9 ± 0.1
22:5ω3	0.4 ± 0.1	0.5 ± 0.1	0.7 ± 0.1	1.3 ± 0.1
22:6ω3	3.2 ± 0.3	4.8 ± 0.6	2.0 ± 0.2	2.5 ± 0.1

[a]The results expressed as the percentage of total fatty acids are means ± SM.
[b]Number in parentheses indicates number of mice.
ND, not detected.

amount of PL (2). This latter effect was most pronounced for those PL containing polyunsaturated fatty acids in C20 and C22, which correlated with a decrease in the incorporation of 2-^{3}H-glycerol into polyunsaturated species of PL (this publication). At the same time, the concentration of precursors (1,2-diacylglycerols) increases markedly without significant modifications in their renewal or in their composition in polyunsaturated fatty acids. Our present results may best be discussed relative to the pronounced metabolic changes that occur in the liver of normal animals during the postnatal period.

Pollak and Harsas (25) observed an increase in the polyunsaturated fatty acids arachidonic (20:4ω6) and docosahexaenoic (22:6ω3) and a decrease in the proportion of oleic acid (18:1) in the PL of liver mitochondria in 3-day-old rats as compared to newborn. Ogino et al. (26) showed that in suckling rats the polyunsaturated species of liver choline glycerophospholipids increased rapidly during the first days of life, in contrast to the decrease in the monoenoic species. We noted similar changes in the liver PL of control suckling mice in our experiments: an increase in arachidonic (20:4ω6) and docosahexaenoic (22:6ω3) acids and a decrease in oleic acid (Tables 1 and 2). These physiological changes in the degree of unsaturation of liver PL fatty acids in the course of development in the first few days of life was markedly affected by daily administration of interferon. Thus, by the sixth to eighth day of life, interferon treatment resulted in a pronounced decrease in the percentage of polyunsaturated fatty acids in liver PL compared to control mice. In fact, the composition of PL fatty acids of these 6- or 10-day-old interferon-treated suckling mice remains similar to that of newborn mice.

The decrease in the polyunsaturated fatty acids in PL in the livers of interferon-treated mice appeared to be a specific effect and did not stem from a general decrease in the availability of polyunsaturated fatty acids. Thus, when one compares the ratio of fatty acid content of liver PL to the total amount of liver fatty acids, it was apparent that, for each fatty acid examined, this ratio was much lower for interferon-treated suckling mice than for control mice (Table 3). Furthermore, the decrease in 18:1 and the accumulation of the 20:4 and 22:6 species in DG appears to be the result of the decreased incorporation of polyunsaturated fatty acids in the PL of interferon-treated mice.

The second major effect of interferon on liver lipid metabolism concerned the amount of liver PL (2). In the neonatal period of several different animal species, selective changes occur in the activity and concentration of microsomal enzymes for the synthesis of liver PL and triacylglycerols (27-31). As shown in Table 4, interferon treatment inhibited the physiological increase in the synthesis of liver PL. When the activity was expressed per 100 mg of protein, the value in 10-day-old control mice was double that of newborn or interferon-treated mice. This effect of interferon on PL synthesis was especially marked for PC and PE. Induction of the synthesis of diacylglycerol-choline-phosphotransferase and diacylglycerol-ethanolamine-phosphotransferase occurs in rats during the first days of life (27,31,32). This increase in the activity of the PC and PE synthesis is probably accompanied by a change in the specificity of the enzymes toward the different molecular species of diacylglycerol (33). In this regard, it has recently been reported that there is a significant increase in the production of liver microsomal PC, with an increasingly higher proportion of unsaturated fatty acids in the perinatal period (26, 34,35). In parallel, there is also an increase in the activity of the PC synthesis by N-methylation of PE (27), a metabolic pathway that produces molecular species with a high content of polyunsaturated fatty acids (22,36,37).

It is also possible that interferon treatment resulted in an inhibition of the reacylation of lysophospholipids formed by hepatic phospholipases (38-40). In fact, the microsomal lysophospholipid acyltransferases, which can remodel PL by transferring fatty acids on C2 of the glycerol of lysophospholipids, show considerable activity toward polyunsaturated acyl-CoA (38-42).

The results in Table 5 suggest that interferon treatment of suckling mice inhibits this change in the molecular specificity of the biosynthetic enzymes. For example, the inhibitory effect of interferon is especially evident in the molecular species of PC and PE, having the greatest percentage of polyunsaturated fatty acids in C20 and C22. The liver content of these molecular species is about half as much in 10-day-old interferon-treated mice (1.66 μmol per liver) as in 10-day-old control mice (3.28 μmol) (Table 4). The inhibition of the synthesis of polyunsaturated PL in interferon-treated mice can be related to the ultrastructural observations of Moss et al. (3,4), who showed that interferon treatment of suckling mice resulted in the rapid appearance of characteristic tubular aggregates associated with the granular endoplasmic reticulum. In the postnatal period, there is also a rapid development and increase in the endoplasmic reticulum, which is the principal site of PL synthesis in hepatocytes (25,43,44). One may suggest that interferon alters the normal development of the endoplasmic reticulum, which would then limit the growth of enzymatic systems necessary for the synthesis of PL, or that interferon inhibits the synthesis of those enzymes necessary for PL synthesis, which, in turn, would limit the construction of new membranes essential for the developing endoplasmic reticulum (32).

Lastly, interferon treatment results in a very marked increase in liver diacylglycerol level. The diacylglycerol accumulation in response to hormonal stimulation has been shown using a variety of cells and hormones (45-47). Diacylglycerol serves as a substrate for lipases or as a precursor in PL and triacylglycerol synthesis. But it can also act as a second messenger and can regulate protein kinase C activity (47-48). The increase in the hepatic diacyglycerol concentration in interferon-treated suckling mice may alter the metabolic response of the liver to different hormonal stimuli in the postnatal period (49). Indeed, Wolfman and Macara (50) have recently observed that the ras-transformed fibroblasts possess elevated diacylglycerol levels and have decreased sensitivity to phorbol ester activation of protein kinase C.

In Daudi cells, which had been prelabeled with [2-$_3$H]-glycerol, Yap et al. (51,52) have observed a very early and transient two- to threefold increase in the concentration of radiolabel in DG of interferon-treated cells. Interferon in concentration exceeding 2 IU/ml

inhibits the division of cells, and the degree of inhibition corresponds closely to the size of the increase in diacylglycerol label (52). In newborn mice, interferon treatment does not result in an inhibition of liver growth (2), and the specific radioactivity of liver DG after injection of (1,3-^{3}H)-glycerol appeared identical between the two groups of mice. In fact, in vivo, the increase in the concentration of DG reflects a more complex action of interferon on the metabolism of lipids in the liver. Interferon probably alters in the newborn mouse the hepatic pathways responsible for formation and utilization of DG.

This ensemble of results, especially the decrease in liver polyunsaturated PL and increase in DG would appear to have consequences for the maturation of hepatocytes in suckling mice. In many respects, the hepatocytes of 10-day-old interferon-treated suckling mice resembled newborn mice biochemically (rather than control 10-day-old littermates). On the other hand, the fact that interferon interferes with the synthesis of certain polyunsaturated species of PL in the liver (especially certain species of polyenoic PC and PE) of suckling mice may lead to a decrease in the general availability of polyunsaturated species of these PL indispensable for the development of other tissues (35,53). This decrease in polyunsaturated fatty acids availability may be related to other interferon-induced lesions, which only become manifest later in life (54-56).

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New Findings in the Fatty Acid Composition of Individual Platelet Phospholipids in Man after Dietary Fish Oil Supplementation

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Nine healthy male volunteers were given 15 Max EPA fish oil capsules providing 2.67 g of eicosapentaenoic acid (EPA, 20:5ω3) and 1.72 g of docosahexaenoic acid (DHA, 22:6ω3) daily for 3 wk. Measurements were taken at baseline, at the end of the fish-oil period, and at 2 and 6 wk postsupplementation. The effect of fish oil on plasma lipids and the fatty acid composition of individual platelet phospholipids was studied. In general, the proportions of 20:5ω3 and 22:6ω3 in platelet phosphoglycerides were substantially increased mainly at the expense of arachidonic acid (AA, 20:4ω6). A large and significant increase in the relative EPA content of phosphatidylcholine (PC) ($P < 0.001$) and phosphatidylethanolamine (PE) ($P < 0.001$) was noted at the end of the 3 wk supplementation. We have also shown for the first time a small but significant ($P < 0.001$) incorporation of EPA in phosphatidylserine (PS). Incorporation of DHA was also detected in PC, PE and PS, whereas the relative AA content of these phospholipids was significantly reduced. Fish oil supplementation led to a significant increase of 22:5ω3 in PS and decreases of 20:3ω6 in PC and 22:4ω6 in PE. Postsupplementation measurements showed a gradual return of all fatty acids to baseline levels. The fatty acid composition of the phosphatidylinositol (PI) fraction remained unchanged throughout the trial period. We conclude that in humans ω3 fatty acids are incorporated into platelet membrane phospholipid subclasses with a high degree of specificity.
Lipids 22, 744–750 (1987).

Both arachidonic acid (AA) and eicosapentaenoic acid (EPA) are substrates for the cyclooxygenase and lipoxygenase enzymes and thus are precursors of eicosanoids, which have a broad spectrum of biological activities and have been implicated in the pathogenesis of atherothrombotic and inflammatory processes (1–3). However, the eicosanoids derived from AA and EPA differ significantly in biological activity. Whereas thromboxane A_3 (TXA_3) is less proaggregatory than thromboxane A_2 (TXA_2) (4,5) and leukotriene B_5 (LTB_5) is less active as a chemotactic compound than leukotriene B_4 (LTB_4) (6–8), prostaglandin I_3 (PGI_3) is as potent as prostaglandin I_2 (PGI_2) as an antiaggregatory agent (4,9). Consequently, a shift of prostanoid formation from the 2- to the 3-series may change the TXA/PGI balance (10) favorably (1).

A number of studies in humans in which a diet has been supplemented with ω3 fatty acids given as either fish meal, fish oil concentrate or purified ω3 fatty acids (11–14) have shown a favorable pattern on blood and platelet lipids (15–17), reduced platelet aggregability (17–18), a prolonged bleeding time (18) and a reduced blood pressure response to pressor hormones (18). Furthermore, dietary EPA is transformed to PGI_3 (9), while TXB_3, the stable metabolite of platelet-derived TXA_3, has been observed in platelets (19).

In order to elucidate further the mode of action of the ω3 fatty acids on platelet reactivity, previous investigators have examined the fatty acid composition of platelet membrane phospholipids (11,14,20–23). These analyses have been confined largely to determining changes in fatty acid composition of total phospholipids or changes in the relative content of AA and EPA. While these are considered the principal fatty acids, there has been a tendency to overemphasize EPA and disregard the possible effects of other ω3 polyunsaturated fatty acids (PUFA) present in fish diets that could also be incorporated into membrane phospholipids and modify subsequent eicosanoid metabolism. For this reason we examined the total fatty acid composition of individual platelet phospholipids of wider range of ω3 PUFA in human subjects taking dietary fish oil supplements.

MATERIALS AND METHODS

Study design. Nine healthy male volunteers, 20 to 45 years of age, who were nonsmokers were asked to withhold all drugs known to interfere with eicosanoid formation or lipid metabolism during the study period of 10 weeks. Two baseline measurements on separate days were taken during the first week, and volunteers were then instructed to take five Max EPA capsules three times daily with meals for 3 wk. This supplement is equivalent to a daily intake of 2.67 g EPA and 1.72 g docosahexalaoic acid (DHA). Further measurements were made at the end of the fish-oil period, as well as 2 and 6 wk postsupplementation.

Materials. One g of Max EPA capsules (PR Scherer Pty Ltd., Melbourne, Australia) contained EPA (17.82%) and DHA (11.49%) as verified by gas liquid chromatography (GLC) (see Table 1). The vitamin A and E content of the Max EPA was measured by Dr. D. S. Petterson using high performance liquid chromatography and tocol as an internal standard. Cholesterol content was determined by Dr. F. Legge utilizing GLC and cycloartenol as an internal standard. Both analyses were carried out at the Western Australian Department of Agriculture. The spary reagent 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt was purchased from Eastman Kodak Co. (Rochester, NY); 14% boron trifluoride in methanol was from BDH Chemical Ltd; authentic standard fatty acid methyl

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Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; TXA_2, thromboxane A_2; TXA_3, thromboxane A_3; LTB_4, leukotriene B_4; LTB_5, leukotriene B_5; PGI_2, prostaglandin I_2; PGI_3, prostaglandin I_3; PUFA, polyunsaturated fatty acid; GLC, gas liquid chromatography; TLC, thin layer chromatography; HDL, high density lipoprotein; LDL, low density lipoprotein.

TABLE 1

Fatty Acid Composition of Max EPA Capsules

Fatty acid	Total fatty acids by weight (%)
14:0	7.54
14:1	0.25
15:0	0.56
16:0	16.26
16:1ω7	8.86
17:0	1.33
17:1 + 17:0Br[a]	1.36
18:0	3.30
18:1ω9	14.06
18:2ω6	1.35
18:3ω3	0.62
20:0	2.16
20:4ω6	1.05
20:5ω3	17.82
22:1ω11	0.69
22:5ω3	2.78
22:6ω3	11.49

[a]Br denotes branched chain.

TABLE 2

Amounts of ω3 Fatty Acids, Vitamins and Cholesterol Provided by 15 g/Day of Max EPA Oil

18:3ω3	0.09 g/day
20:5ω3	2.67 g/day
22:5ω3	0.42 g/day
22:6ω3	1.72 g/day
Total ω3	4.90 g/day
Vitamin A	1380 μg/day
Vitamin E	7890 μg/day
Cholesterol	57 mg/day

esters and phospholipids were from Sigma Chemical Co. (St. Louis, MO). Thin layer chromatography (TLC) was carried out on silica gel 60 F_{254}, precoated aluminum sheets (Merck Art. 5554). GLC was performed on a Packard Model 437 Gas Chromatograph equipped with a Packard Model 604 Recording Data Processor.

Platelet preparation and lipid extraction. Blood (20 ml) from unfasted volunteers was obtained by venipuncture and collected into EDTA (1 mg/ml), then briefly shaken and centrifuged at 110 × g for 15 min at room temperature. Platelet pellets were prepared at room temperature as follows: platelet-rich plasma was removed and centrifuged at 1000 × g for 15 min, and the platelet-poor plasma was frozen (-20 C) until analysis. The platelet plug was resuspended in EDTA/NaCl buffer (pH 7) and recentrifuged. Washed platelets were extracted with $CHCl_3/CH_3OH$ (2:1v/v, 5 ml) containing 50 mg/l of the antioxidant butylated hydroxytoluene, the sample was centrifuged at 1000 × g for 5 min, and the organic phase was evaporated under vacuum yielding a crude lipid extract.

TLC and fatty acid analysis. Phospholipid subclasses were separated from total lipid extracts by TLC using a solvent system of ethyl acetate/N-propanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:13 :9,v/v/v/v/v) (24). TLC plates were sprayed with the reagent 8-hydroxy-1,3,6-pyrenetrisulfonic acid trisodium salt (25) (10 mg/100 ml methanol), allowed to dry for several minutes and visualized under UV light (254 nm and 366 nm). The zones containing phosphatidyl ethanolamine (PE), -inositol (PI), -serine (PS) and -choline (PC), as determined by comparison with authentic standards, were scraped off, and fatty acid methyl esters were prepared by treatment of samples with 14% boron trifluoride in methanol at 100 C for 30 min (26). After dilution with water, methyl esters were extracted with hexane, dried under N_2 and then dissolved in hexane (5 μl) for GLC analysis. The methyl esters were separated on a BP 20 vitreous silica column (0.53 mm × 12 m) (Scientific Glass Engineering, Melbourne, Australia), temperature-programmed from 180 C to 220 C at 5 C/min with hydrogen as carrier gas and a split ratio of 50:1. Peaks were identified by comparison with a known standard mixture of fatty acid methyl esters. The amounts of individual fatty acids were calculated as relative percentage with the evaluated fatty acids set as 100%.

Plasma lipids. Lipids from platelet-poor plasma frozen at -20 C were assayed as follows: cholesterol and triglyceride were determined enzymatically on an Abbott ABA-100 analyzer using reagents from Abbott and standardized with serum-based calibrators, the values of which were traceable to the Centers for Disease Control (Atlanta, GA). The coefficients of variation were 1.5 and 2.0%, respectively. Plasma high density lipoprotien (HDL) cholesterol was assayed on a heparin-manganese chloride supernatant (27) (final manganese concentration 0.091 mol/l) with addition of 4 mM EDTA to the Abbott cholesterol reagent (28). HDL subfractions, HDL_2-C and HDL_3-C, were measured by the double precipitation method of Gidez et al. (29) (final dextran sulphate concentration 0.13%). Statistics calculated on 42 separate runs using a subject's aliquoted frozen (-70 C) plasma were as follows: mean total HDL cholesterol, 1.61 mmol/l with standard deviation 0.057 mmol/l and coefficient of variation 3.53%; mean HDL_3 cholesterol, 0.734 mmol/l with standard deviation 0.063 mmol/l and coefficient of variation 8.63%; and mean HDL_2 cholesterol 0.874 mmol/l, standard deviation 0.057 mmol/l and coefficient of variation 6.53%. Low density lipoprotein cholesterol (LDL-C) was calculated from the Friedewald formula (30).

Statistics. Results are expressed as the mean and standard error of the mean (SEM). One-way analysis of variance (ANOVA) using repeated measures was used to test for variations between visits for individual fatty acids. Differences between any two visits within a particular fatty acid were established by the Newman-Keuls test.

RESULTS

The fatty acid composition of the Max EPA capsules is shown in Table 1. The amounts of ω3 fatty acids,

TABLE 3

Effect of Max EPA Oil Intake (3 Wk, 15 g/Day) on Fatty Acid Composition of Phosphatidylserine in Platelets

	% Composition at each subject visit				
Fatty acid	1[a]	2[a]	3[b]	4[c]	5[c]
16:0	7.46 ± 1.52	6.04 ± 0.84	6.85 ± 0.53	8.70 ± 0.63	7.40 ± 0.72
18:0	36.23 ± 1.44	35.45 ± 0.98	35.76 ± 0.78	35.26 ± 1.27	32.99 ± 1.79
18:1	21.55 ± 0.80	22.39 ± 0.69	21.07 ± 1.00	20.46 ± 1.11	23.80 ± 1.01
18:2	2.07 ± 0.56	1.78 ± 0.57	1.99 ± 0.43	2.34 ± 0.57	2.85 ± 0.58
20:4	28.40 ± 1.37	28.33 ± 1.25	23.55 ± 1.05*	26.67 ± 1.70	26.29 ± 1.25
20:5	0.39 ± 0.18	0.81 ± 0.20	1.68 ± 0.26***	1.17 ± 0.16	0.61 ± 0.16
22:4	0.73 ± 0.16	1.21 ± 0.08	0.93 ± 0.16	1.06 ± 0.18	1.22 ± 0.29
22:5	1.32 ± 0.24	1.79 ± 0.21	2.82 ± 0.26**	2.05 ± 0.41	1.82 ± 0.45
22:6	2.30 ± 0.14	2.13 ± 0.19	4.87 ± 0.49***	3.48 ± 0.51*	2.73 ± 0.31

Values are mean ± SEM of 9 subjects. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ for individual fatty acids as compared to vist 2.
[a]Visits 1 and 2, baseline measurements.
[b]Visit 3, at the end of the 3-week supplementation period.
[c]Visits 4 and 5, measurements taken 2 and 6 wk postsupplementation.

TABLE 4

Effect of Max EPA Oil Intake (3 Wk, 15 g/Day) on Fatty Acid Composition of Phosphatidylethanolamine in Platelets

	% Composition at each subject visit				
Fatty acid	1[a]	2[a]	3[b]	4[c]	5[c]
16:0 DMA[d] + 16:0	9.43 ± 0.51	9.18 ± 0.69	12.19 ± 1.58	10.42 ± 1.05	10.12 ± 1.12
16:1 DMA	4.11 ± 0.97	6.17 ± 1.47	4.60 ± 0.78	4.61 ± 0.80	5.84 ± 1.68
18:0 DMA + 18:0	21.73 ± 1.52	19.58 ± 1.27	19.73 ± 1.43	21.44 ± 0.88	20.59 ± 1.26
18:1	6.69 ± 0.43	5.71 ± 0.50	6.15 ± 0.47	6.61 ± 0.59	8.26 ± 0.84**
18:2	2.69 ± 0.32	2.05 ± 0.20	2.41 ± 0.43	2.05 ± 0.12	3.02 ± 0.54
20:4	40.22 ± 0.97	41.57 ± 1.13	33.64 ± 1.12***	37.41 ± 0.58*	39.55 ± 1.44
20:5	1.35 ± 0.13	1.20 ± 0.23	7.57 ± 0.52***	3.04 ± 0.38***	2.17 ± 0.34
22:4	4.58 ± 0.20	4.77 ± 0.43	2.31 ± 0.28***	3.39 ± 0.25**	3.53 ± 0.28**
22:5	4.46 ± 0.34	4.73 ± 0.25	5.53 ± 0.36	5.37 ± 0.13	4.23 ± 0.37
22:6	4.05 ± 0.38	4.12 ± 0.22	5.65 ± 0.29**	5.40 ± 0.32**	3.71 ± 0.40

Values are mean ± SEM of 9 subjects. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ for individual fatty acids as compared to visit 2.
[a]Visits 1 and 2, baseline measurements.
[b]Visit 3, at the end of the 3-week supplementation period.
[c]Visits 4 and 5, measurements taken 2 and 6 wk postsupplementation.
[d]DMA, dimethylacetal.

vitamins A and E, and cholesterol provided by the fish oil are shown in Table 2. With respect to the ω3 fatty acids, the major contributors were 20:5 (17.82% of the total fatty acids by weight) and 22:6 (11.49%), with minor contributions from 18:3 (0.62%) and 22:5 (2.78%). Vitamin E is added to the capsules as an antioxidant.

The fatty acids shown in each of the phospholipids (Tables 3-6) were those observed within the detectable limit of the gas chromatograph and clearly demonstrate a variation in the fatty acid distribution in phospholipid subclasses. All comparisons within fatty acids are relative to the second baseline visit, which in all cases did not differ statistically from the first.

In general, after 3 wk of taking the oil, the proportions of 20:5ω3 and 22:6ω3 were substantially increased in platelet phosphoglycerides, mainly at the expense of ω6 polyunsaturated fatty acids, and in particular 20:4ω6 (Tables 3-5). The one exception was PI, which showed no significant changes in any of the fatty acids during the trial period (Table 6).

At the end of the supplement, the PS fraction showed significant increases in the levels of 20:5ω3 and 22:6ω3, which together constitute about 30% of the capsule content by weight (Table 3). In addition, a significant increase in 22:5ω3 and a fall in 20:4ω6 ($P < 0.05$) were observed. The levels of 20:4ω6, 20:5ω3, 22:5ω3 and

TABLE 5

Effect of Max EPA Oil Intake (3 Wk, 15 g/Day) on Fatty Acid Composition of Phosphatidylcholine in Platelets

	% Composition at each subject visit				
Fatty acid	1[a]	2[a]	3[b]	4[c]	5[c]
16:0	27.29 ± 0.55	27.06 ± 0.76	28.64 ± 0.84	27.56 ± 0.96	28.23 ± 0.96
16:1	0.71 ± 0.20	0.94 ± 0.11	0.67 ± 0.16	0.95 ± 0.35	0.79 ± 0.22
18:0	15.69 ± 0.40	14.53 ± 0.81	14.51 ± 1.13	15.02 ± 1.12	12.45 ± 0.77
18:1	23.40 ± 0.67	22.73 ± 0.32	24.15 ± 0.72	23.69 ± 0.59	25.97 ± 0.62**
18:2	8.37 ± 0.53	8.92 ± 0.77	8.29 ± 0.97	9.31 ± 1.15	8.54 ± 0.57
20:1	1.38 ± 0.16	2.31 ± 0.43	1.74 ± 0.46	1.35 ± 0.16	1.99 ± 0.40
20:3	1.56 ± 0.29	2.05 ± 0.15	1.05 ± 0.17**	1.43 ± 0.14	1.60 ± 0.21
20:4	15.61 ± 0.66	16.82 ± 1.07	11.20 ± 0.61***	13.41 ± 1.06**	15.28 ± 0.36
20:5	1.03 ± 0.18	0.85 ± 0.17	4.33 ± 0.33***	2.39 ± 0.34***	1.73 ± 0.28*
22:4	0.85 ± 0.11	0.71 ± 0.07	0.57 ± 0.08	0.86 ± 0.13	0.75 ± 0.08
22:5	1.26 ± 0.10	1.11 ± 0.13	1.54 ± 0.10	1.50 ± 0.17	1.12 ± 0.07
22:6	1.40 ± 0.21	1.57 ± 0.26	3.01 ± 0.50**	2.49 ± 0.33*	1.53 ± 0.28

Values are mean ± SEM of 9 subjects. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ for individual fatty acids as compared to visit 2.
[a]Visits 1 and 2, baseline measurements.
[b]Visit 3, at the end of the 3-week supplementation period.
[c]Visits 4 and 5, measurements taken 2 and 6 wk postsupplementation.

TABLE 6

Effect of Max EPA Oil Intake (3 Wk, 15 g/Day) on Fatty Acid Composition of Phosphatidylinositol in Platelets

	% Composition at each subject visit				
Fatty acid	1[a]	2[a]	3[b]	4[c]	5[c]
16:0	15.62 ± 1.59	17.70 ± 1.25	17.69 ± 1.81	15.66 ± 1.60	16.11 ± 1.29
18:0	33.07 ± 1.72	30.88 ± 1.26	32.86 ± 1.77	34.29 ± 1.33	32.23 ± 1.72
18:1	14.43 ± 1.24	12.96 ± 1.71	12.23 ± 1.85	14.81 ± 1.36	14.53 ± 1.68
20:4	36.48 ± 1.71	38.16 ± 1.73	36.53 ± 1.88	35.30 ± 1.49	36.82 ± 2.50

Values are mean ± SEM. ANOVA between visits within individual fatty acids: no significant difference.
[a]Visits 1 and 2, baseline measurements.
[b]Visit 3, at the end of the 3-week supplementation period.
[c]Visits 4 and 5, measurements taken 2 and 6 wk postsupplementation.

22:6ω3 all showed a return to basal levels within 2 wk of ceasing supplementation, although that of 22:6ω3 remained significantly higher compared with baseline values.

The platelet PE fraction showed a significant fall in 20:4ω6 ($P < 0.001$) at the end of the supplementation period and a gradual return to pretreatment levels 2 wk after the supplement had been withdrawn (Table 4). In a similar way, the level of the chain elongation product of 20:4ω6 (22:4ω6) fell significantly during the intervention period, gradually rising afterward, although remaining lower at 2 and 6 wk. The relative proportions of the ω3 fatty acids 20:5, 22:5 and 22:6 all increased during the supplement. The largest increase was in 20:5ω3 ($P < 0.001$) and to a lesser extent 22:6ω3. The level of 22:5ω3 was also slightly increased, but this was not significant. Both 20:5ω3 and 22:6ω3 remained high ($P < 0.001$ and $P < 0.01$, respectively) 2 wk after Max EPA but had reached basal levels after 6 wk.

Analysis of the PC fraction showed a five fold increase in 20:5ω3 and a two fold rise in 22:6ω3 (Table 5). Both fatty acids remained significantly high 2 wk after oil supplementation and that of 20:5ω3 was still high after 6 wk. A small but statistically insignificant rise in 22:5ω3 was observed during the supplementation period. The levels of the two ω6 fatty acids, 20:3 and 20:4, were both lowered significantly during fish oil supplementation. That of 20:3ω6 had returned to baseline within 2 wk of ending the supplement, while that of 20:4ω6 had reached basal levels by the sixth week. It is significant to note that the fatty acid

TABLE 7

Effect of Max EPA Oil Intake (3 Wk, 15 g/Day) on Plasma Lipids

	% at each subject visit		
	2[a]	3[b]	5[c]
Cholesterol (mmol/l)	5.12 ± 0.32	5.34 ± 0.36	5.08 ± 0.39
Triglycerides (mmol/l)	1.18 ± 0.23	1.15 ± 0.25	1.11 ± 0.27
HDL cholesterol (mmol/l)	1.11 ± 0.16	1.16 ± 0.13	1.25 ± 0.15
HDL_2 cholesterol (mmol/l)	0.68 ± 0.12	0.78 ± 0.12	0.72 ± 0.13
HDL_3 cholesterol (mmol/l)	0.44 ± 0.04	0.38± 0.04	0.59 ± 0.04
LDL cholesterol (mmol/l)	3.46 ± 0.36	3.65 ± 0.35	3.33 ± 0.39

Values are mean ± SEM. ANOVA between visits: no significant difference. HDL, high density lipoprotein; LDL, low density lipoprotein.
[a]Visit 2, baseline measurement.
[b]Visit 3, at the end of the 3-wk supplementation period.
[c]Visit 5, 6 wk postsupplementation.

20:3ω6 is the immediate precursor of 20:4ω6. The proportion of 22:4ω6 was not significantly altered during the trial.

Plasma cholesterol, triglycerides and lipoproteins were not significantly altered by the fish oil (Table 7).

DISCUSSION

It has been suggested that the low incidence of cardiovascular disease in Eskimos may be related to their diet, which consists mainly of marine fish (11-13, 31,32). Subsequent evidence indicates that this may be due to the high intake of polyunsaturated fatty acids of the ω3 series and, in particular, EPA and DHA, both of which constitute a large proportion of the fish oil. The diet may consequently lead to a synthesis of the 3-series prostanoids replacing the platelet-reactive prostanoids of the 2-series (1,17). The resultant shift in balance of proaggregatory to antiaggregatory prostanoids would then lead to a reduced thrombotic tendency.

Recent attention has thus turned to supplementation of Western diets with fish oils to elucidate their effect on blood lipids, platelet function and eicosanoid production (11-13, reviews), factors which are implicated in the pathogenesis of cardiovascular disease. However, in many cases, different fish containing varying types and amounts of fatty acids have been used, making comparisons of results between studies difficult. Although fish oil is a readily available source of EPA, significant amounts of DHA and other fatty acids are present, so it is not possible to attribute all the effects observed to EPA alone. In particular, DHA had been shown in vitro (33,34) and more recently in vivo (22) to inhibit platelet aggregation. Since platelet reactivity may be dependent on the fatty acid composition of its membrane, a number of studies have examined changes in fatty acid content following dietary fish oil supplementation (11,14, 20-23). Many of these studies have been limited, reporting either changes in fatty acid composition of total platelet phospholipids or changes in the relative content of several of the major fatty acids. The principal problems have been the time required and the difficulty in cleanly separating the major phospholipid subclasses. In a recent study in which volunteers supplemented their diet with 25 ml/day of cod liver oil for 5 mo, individual phospholipids were separated. However, of the long chain fatty acids, only EPA and DHA from the ω3 series and AA from the ω6 series were reported (20). Similarly, a previous study had reported total fatty acid composition of PE and PC only. Whereas PS and PI were isolated as one fraction (23). To our knowledge, a detailed analysis of total fatty acid composition of *all* the individual phospholipid subclasses has not been undertaken, warranting this investigation in normal human subjects taking moderate amounts of Max EPA fish oil for 3 wk.

In the present trail, we have been able to obtain complete separation of PE, PC, PS and PI by utilizing one-dimensional TLC and subsequently visualizing the bands with the aid of a reagent that is highly UV-active (see Materials and Methods). The method used is less time-consuming than the often favored two-dimensional TLC; it also is very sensitive and leads to reproducible separation of all phospholipid subclasses.

In platelet PE and PC, EPA increased from control values of 1.20% (PE) and 0.85% (PC) to 7.5% (PE) and 4.33% (PC) during fish oil administration (Tables 4 and 5). At the same time, the levels of DHA also rose from 4.12% to 5.65% in PE and from 1.57% to 3.01% in PC, while both PE and PC showed significant falls in AA content. These findings within PE and PC agree with previous observations (20-23). Interestingly, the level of 22:4ω6 fell significantly in PE from 4.77% to 2.31% for the same period, suggesting a decreased synthesis from its immediate precursor, AA. In light of the fall in AA, the result is not unexpected. Post-oil supplementation measurements show a gradual rise in 22:4ω6 in PE with a significant difference persisting at 6 wk (visit 5), while AA showed a more rapid return to basal levels.

These results may reflect a gradual reactivation of

the enzymes involved in the chain elongation of 20:4ω6 to 22:4ω6. The proportion of 22:4ω6 in PC was, however, considerably lower and not affected by the fish oil, which may indicate a preferential incorporation of this fatty acid into PE. A similar fall in 22:4ω6 has been observed in both PE and PC after 6 wk of dietary supplementation with cod liver oil (23). The proportion of 20:3ω6, the immediate precursor of AA, fell from 2.05% to 1.05% ($P < 0.01$) in PC during the fish oil intake. This may indicate a reduction in its synthesis from 18:2ω6 and may have contributed to the fall in AA in PC. It is interesting to note the significant rise ($P < 0.01$) in the level of oleic acid (18:1ω9) in both PE and PC 6 wk after the cessation of the supplement. However, it is doubtful whether this can be attributed to the fish oil; it may merely reflect a change of diet by several of the participants after ceasing the supplement.

The PS fraction provided some interesting and novel results (Table 3). The increase in 20:5ω3 ($P < 0.001$) and 22:5ω3 ($P < 0.01$), with a concomitant reduction in 20:4ω6, has not previously been reported and may be important in our understanding of the mode by which fatty acid incorporation occurs. All three fatty acids showed a rapid return, and within 2 wk of ending the supplement they were not different from baseline. The increase in the proportion of 22:5ω3 in PS was large considering the small amount of this fatty acid provided by Max EPA; this would suggest that a portion of the EPA in the diet is chain-elongated to 22:5ω3 and subsequently preferentially incorporated into the PS fraction. Small but insignificant rises in the level of 22:5ω3 were also noted in PE and PC. These results are in contrast with a recent study in which 6 g/day of purified EPA given for 6 days resulted in minimal changes in 22:5ω3 in the first 24 hr, which is expected if it is synthesized from EPA, before rising significantly in PE and PC only (22). The study also suggests that dietary EPA was not metabolized to DHA, indicating that the increase in DHA that we have observed in PS, PE and PC is directly obtained from Max EPA. In another study, a significant decrease in 22:5ω3 was observed in a combined fraction of PS + PI after administering dietary cod liver oil (23). The investigators were, however, unable to confirm which phospholipid had contributed to the fall in 22:5ω3. The significance of the small quantity of 22:5ω3 in PS remains to be elucidated.

None of the other fatty acids examined in the phospholipid subclasses were altered by the fish oil during our trial. We were unable to confirm a previous finding of a significant reduction in the 18:2ω6 content of PE after 4 wk of Max EPA (21).

In accordance with previous investigations, there were no appreciable changes in the fatty acid composition of PI during fish oil supplementation (Table 6) (11,13,20-22). This failure to incorporate EPA, even when the fatty acid is provided in purified and high doses, reflects a tightly controlled system (22). The suggestion that the phospholipid fatty acid composition of blood cells is predetermined during their formative stages (17,20,22) would certainly be compatible with the observation for PI, but not for PE, PC or PS. A second regulatory mechanism proposed involves a "fine tuning" of the fatty acid compositions, and this does appear to operate in the three latter phospholipids (20). It is worth noting that both neutral lipids and the polar lipids of marine fish are rich in ω3 PUFA, with a ratio of ω3/ω6 PUFA of 10-15:1 (35). It has been shown recently that the composition of PI from marine fish is very similar to that in terrestrial mammals, whereas the other phospholipids contain large amounts of ω3 PUFA obtained from the diet (35). These observations would support the proposal that fatty acid composition of PI is strictly controlled and not responsive to dietary manipulation. The apparent inability to obtain incorporation into PI during fish oil supplementation may be of importance, as this phospholipid is thought to be a major source of AA, which is the substrate for both the cyclooxygenase and lipoxygenase enzymes.

It is clear that in vivo the mechanisms that regulate the fatty acid composition in different platelet phospholipid subclasses differ from those that operate in vitro. In the latter, it has been repeatedly shown that radiolabeled EPA and DHA are rapidly and significantly incorporated into all phospholipid subclasses, including PI, and this is independent of the presence of labeled AA (20,34,36-38).

The most consistent effect of fish oils on blood lipids has been a fall in triglycerides (11,14). Furthermore, in a trial in which men consumed varying amounts of Max EPA for 3 wk periods, plasma triglycerides fell proportionally to the supplemental dosage (39). The effect of dietary fish oils on the plasma total cholesterol levels appears to depend on both the kind of dietary treatment provided and the quantity consumed (11,14). Significant changes were observed only in feeding trials that used very high doses of fish oil and Max EPA, resulting in lower total cholesterol (11, 14,39). Changes in HDL cholesterol concentration have not been frequently observed. An increase in HDL cholesterol was found only after a 20 g/day Max EPA supplementation for 3 wk (39). Variable changes in LDL cholesterol have been observed after supplementation with fish oils.

In conclusion, we have shown that fish oil supplements cause marked changes in the fatty acid composition of PE and PC but not in the PI fractions observations that agree with previous findings (11,14, 20-22). We have also noted, for the first time, that EPA incorporation may occur into the PS fraction. Our findings reinforce the hypothesis that in vivo fatty acid incorporation into platelet membrane phospholipid subclasses occurs with a high degree of specificity, which may reflect the operation of tightly regulated control mechanisms (17,20,22). At this stage, the importance of these observations is uncertain, but it has been suggested that it may by necessary to modify fatty acid composition of specific lipid pools within the platelet membrane in order to alter eicosanoid production (38). The extent to which the membrane composition must be altered in order to affect function also remains unknown. However, in view of the current results, the changes in a wider range of fatty acids within individual phospholipids need to be considered in interpreting the molecular and functional effects of dietary fish oil.

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METHODS

Rapid Isolation of Microsomes for Studies of Lipid Peroxidation

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Conventional isolation of microsomes by high-speed centrifugation from isotonic sucrose requires exposure to air for several hours, leading to the formation of low levels of lipid peroxidation products. Sucrose interferes in protein and malondialdehyde assays and provides no protection against lipid peroxidation during workup. A new procedure for the purification of microsomes from rat liver substitutes mannitol (a hydroxyl radical scavenger) for sucrose and takes advantage of the properties of morpholinopropane sulfonic acid (MOPS) buffer and triethylenetetramine to provide protection against lipid peroxidation during the rapid (less than one hour) workup and subsequent low-temperature storage. The microsomal fractions prepared by the proposed method are free of detectable mitochondrial contamination and at least as pure overall as those prepared by the conventional method, but they have higher glucose-6-phosphatase and laurate hydroxylase activities and significantly less malondialdehyde than conventional microsomes at the time isolation is complete. Laurate hydroxylase activity is more stable during frozen storage in mannitol medium. The kinetics of lipid peroxidation in vitro are quite different for microsomes prepared by the two methods.
Lipids 22, 751–756 (1987).

The difficulties associated with quantitative monitoring of lipid peroxidation in vivo generally preclude studies of the chemical mechanisms involved (1,2). One of the most widely used model biological systems in which such studies have been performed is a suspension of hepatic microsomes, with peroxidation of the membrane lipids supported either nonenzymatically in the presence of ascorbate or enzymatically in the presence of NADPH. Some form of chelated iron is usually supplied as an accelerator of the process, and a variety of products may be measured to follow the course of the complex sequence of reactions involved. The overall process may be indicated by disappearance of polyunsaturated fatty acids or by production of conjugated double bonds, lipid peroxides or hydroperoxides, volatile hydrocarbons, carbonyls or compounds that react with thiobarbituric acid (TBA) to yield a red pigment. Under appropriate conditions (3), the TBA assay may be specific for the quantitative measurement of malondialdehyde (MDA).

A previous publication from this laboratory has emphasized the importance of zero-time controls in studies of lipid peroxidation in microsomes (3). We have always observed lipid peroxidation products to some extent in microsomes prepared by conventional high-speed centrifugation, prior to their incubation. "Conventional" microsomes generally require several hours for isolation and washing, during which time they are exposed to oxygen in the air for at least the first half of the isolation and are in the presence of whatever metal ions may be present in the tissue homogenate. Chelators and/or antioxidants can be included in the homogenate but may be difficult to remove when the microsomes are subsequently to be studied.

We have tended to prepare liver homogenates in 0.25 M sucrose, since many of the products of lipid peroxidation are rapidly metabolized by mitochondria, and we have been unable to prepare microsomes adequately free of mitochondria if ionic media were used in the homogenization. Unfortunately, sucrose can interfere with the TBA assay under some conditions (3) and clearly, in itself, provides no protection against lipid peroxidation during the isolation of the microsomes. The present study was performed in order to develop a more rapid, protective method of microsome isolation yielding a product suitable for studies of lipid peroxidation in vitro.

MATERIALS AND METHODS

Inorganic chemicals used in this study were from Fisher Scientific Co. (Raleigh, NC). Organic chemicals were from Aldrich Chemical Co. (Milwaukee, WI), and biologicals were from Sigma Chemical Co. (St. Louis, MO).

Absorption spectra were scanned using a Beckman DU-7 spectrophotometer with a 0.5-nm slit. Gas chromatography of fatty acid methyl esters (made by transesterification of lipid extracts with boron trifluoride-methanol, ref. 4) was done on a Varian 1200 gas chromatograph using EGSS-X and SE-52 packed columns and an Adalab data system for peak area measurements. Components were identified by correspondence of retention indices with those of known standards from Applied Science Labs. (State College, PA).

Protein in suspensions of microsomes was measured by a modification of the method of Lowry et al. (5) as described previously (6). Cytochrome P-450 was estimated by the method of Omura and Sato (7). Enzymes glucose-6-phosphatase, acid phosphatase, cytochrome C oxidase, 5′-nucleotidase, urate oxidase and laurate hydroxylase were assayed by published methods (8–13). Total microsomal lipid was determined on a weight basis following extraction and washing by the method of Folch et al. as described previously (3). RNA was determined as described by Schneider (14), phospholipid by Bartlett's method (15) and total cholesterol by a ferric chloride method (16). Production of MDA and other thiobarbiturate-reactive substances was monitored as described previously (3). H_2O_2 was monitored by a peroxidase-scopoletin assay (2).

Microsomal suspensions were incubated with NADPH, ferrous sulfate and ADP to promote lipid peroxidation as described elsewhere (3). Controls included incubation mixtures to which trichloroacetic acid (TCA) was added before NADPH (zero-time controls), mixtures lacking

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Abbreviations: TBA, thiobarbituric acid; MDA, malondialdehyde; TCA, trichloroacetic acid; MOPS, morpholinopropane sulfonic acid; TET, triethylenetetramine hydrochloride.

NADPH and mixtures lacking microsomes. Measurements included fatty acid compositions of the incubation mixtures, TBA reactivity (3), conjugated double bonds (17) and lipid hydroperoxides (thiocyanate method, ref. 18).

For electron microscopy, microsomal pellets were held in Fowler's fixative at 4 C overnight, rinsed at 4 C overnight in 0.2 M phosphate pH 7.4, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded ethanol and embedded in Epon. Thin sections (60-90 nm) were cut using a Sorvall MT-1 ultramicrotome and were stained with 5% uranyl acetate/2.7% lead citrate.

Micrographs were taken using a Phillips model 400 electron microscope.

PREPARATION OF MICROSOMES

Conventional high-speed method. One-g portions of diced liver from male CD rats (Charles River, ca. 200 g body weight, fasted overnight prior to death) were homogenized in four volumes of freshly prepared 0.25 M sucrose using a Teflon-glass homogenizer driven by a Wheaton Instruments #903475 stirring motor at gradually increasing speed up to full (five strokes total). The homogenates were centrifuged at 12,000 × g for 10 min in a Sorvall SS34 rotor (4 C), after which the supernates were centrifuged at 100,000 × g for 1 hr in a Ti-60 rotor. The microsomal pellets were resuspended in fresh 0.25 M sucrose and repelleted as above. The final pellets were resuspended in 0.25 M sucrose to 10 mg of protein per ml and were either assayed immediately or were quick-frozen with liquid nitrogen for storage at −70 C.

New method. Four solutions were prepared: wash solution—0.25 M mannitol; homogenizing medium—0.25 M mannitol containing 0.025 M morpholinopropane sulfonic acid (MOPS) buffer pH 7.4 and 0.2 mM triethylenetetramine hydrochloride (TET); dilutant—0.0125 M mannitol containing 0.1 mM TET, 8 mM calcium chloride, pH 7.5; and storage medium—0.25 M mannitol containing 0.025 M MOPS pH 7.4, freshly bubbled with helium to deaerate.

All media were used ice-cold. One-g portions of liver were diced in wash solution, drained and homogenized as for the conventional method, but in the homogenizing medium described. After centrifugation at 12,000 × g for 10 min, the supernate was filtered through a loose plug of glass wool to remove floating fat and again centrifuged at 12,000 × g for 10 min. This supernate was diluted with four volumes of dilutant, stirred with a glass rod for 30 sec and centrifuged for 10 min at 1000 × g (4 C). The pellet was suspended in 2.5 ml of storage medium with gentle stirring to disperse clumps and was either assayed immediately or frozen with liquid nitrogen for storage at −70 C.

RESULTS

Electron micrographs of the preparations of microsomes are shown in Figure 1. The high-speed microsomes are more tightly packed and contain a large number of free ribosomes. The low-speed microsomes are much less tightly packed, but all of the visible ribosomes are either bound to membrane vesicles or clumped together. The micrographs closely resemble those published by Kamath and Rubin (19) and by Baker et al. (20). We have not seen intact mitochondria in either type of preparation.

Composition of and enzyme activity associated with freshly prepared microsomes are summarized in Table 1. All quantitative data in the tables represent triplicate determinations on each of at least three independent preparations of microsomes by each preparative method. In all cases, comparisons are between microsomes prepared by the two methods at the same time from portions of liver from the same rat and are based on two-tailed t-tests with df = 5.

The yield of protein was lower by the new method than by the conventional high-speed method. The ratio of RNA to protein was higher by the new method, but the difference reflected the lower amount of protein. Phospholipid was lower by the new method, but the difference was not statistically significant because of a high standard deviation for conventional microsomes. The ratio of cholesterol to phospholipid was similar for the two methods, as was the concentration of cytochrome P-450.

Acid phosphatase, taken as indicative of contamination by lysosomes, was identical for the two methods. 5'-Nucleotidase, a small amount of which is always found even in the most highly purified microsomes, but which is generally taken as indicative of the amount of contamination by fragments of the plasma membrane, was significantly higher ($P < 0.05$) in the high-speed microsomes. Levels of urate oxidase, a marker for peroxisomes, were quite variable from one preparation to another. The mean was higher for microsomes prepared by the new method, but the difference was not statistically significant. Cytochrome C oxidase, a mitochondrial marker, was extremely low in both preparations.

Highly significant differences ($P < 0.01$) were seen in mean levels of two rather fragile microsomal enzymes, glucose-6-phosphatase and laurate hydroxylase, with the activities being higher in the microsomes prepared by the new method. The differences in glucose-6-phosphatase were much less when microsomes that had been stored at −70 C were compared (Table 2). Laurate hydroxylase was more stable in frozen "new-method" microsomes than in conventional microsomes.

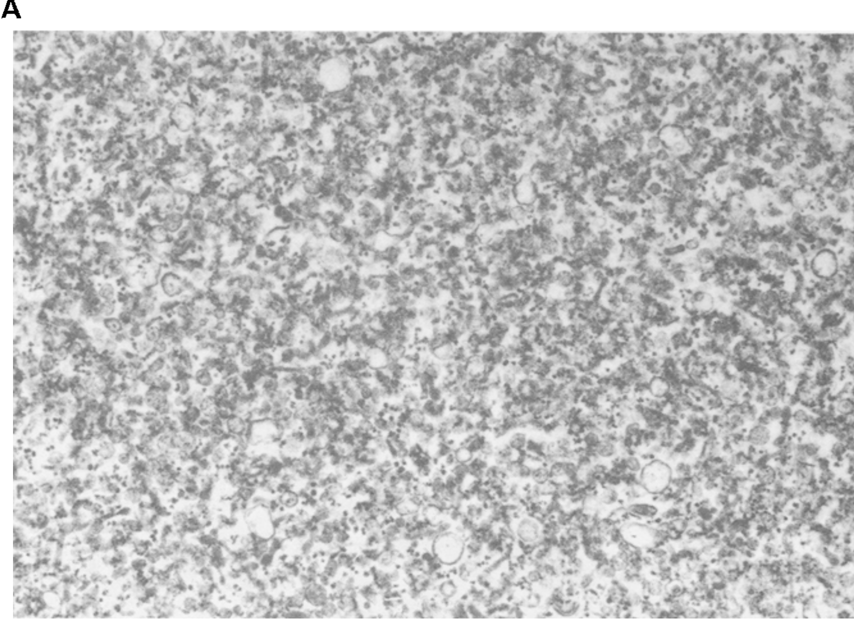
TABLE 1

Properties of Fresh Microsomes

Parameter	Conventional method	New method
Yield of protein[a]	31.4 ± 0.72	25.0 ± 2.1[i]
RNA/protein[b]	0.16 ± 0.003	0.25 ± 0.046[h]
Phospholipid/protein[b]	0.41 ± 0.068	0.33 ± 0.033
Cholesterol/phospholipid[c]	0.134 ± 0.014	0.146 ± 0.006
Cytochrome P-450[d]	0.81 ± 0.019	0.82 ± 0.027
Glucose-6-phosphatase[e]	0.126 ± 0.006	0.145 ± 0.008[i]
Acid phosphatase[e]	0.018 ± 0.002	0.018 ± 0.003
5'-Nucleotidase[f]	6.25 ± 0.42	4.91 ± 0.38[h]
Cytochrome oxidase[e]	0.022 ± 0.004	0.026 ± 0.004
Laurate hydroxylase[g]	1.07 ± 0.065	1.31 ± 0.037[i]
Urate oxidase[e]	3.17 ± 0.38	4.65 ± 1.64

[a]mg/g liver.
[b]mg/mg.
[c]μmol/μmol.
[d]nmol/mg protein.
[e]μmol/min/mg protein.
[f]μmol/hr/mg protein.
[g]nmol/min/mg protein.
[h]$p < 0.05$.
[i]$p < 0.01$.

A



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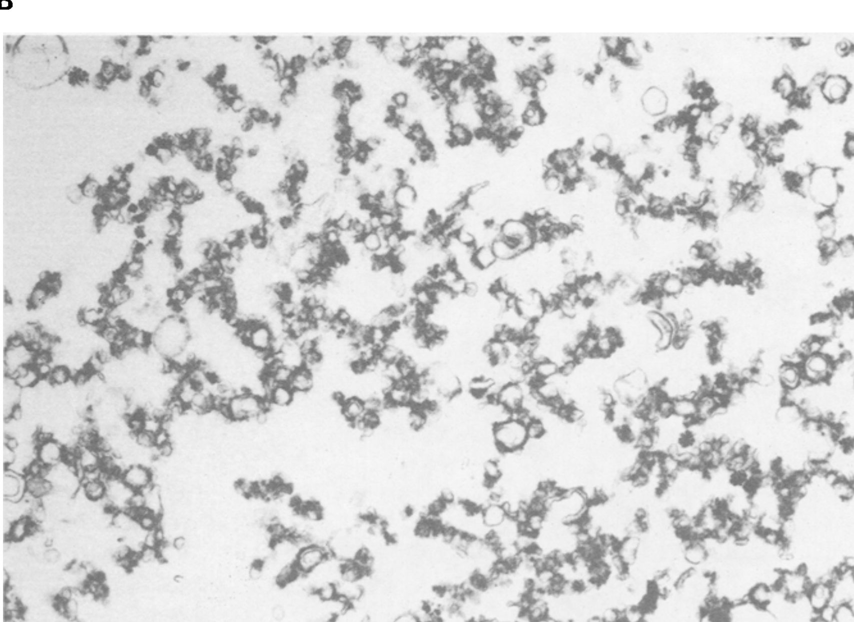

FIG. 1. Electron micrographs of glutaraldehyde-fixed, osmium postfixed, lead-stained pellets of (A) microsomes prepared by high-speed (conventional) centrifugation in sucrose-based medium and (B) microsomes prepared by calcium-precipitation from mannitol-based medium. Initially × 25,000.

TABLE 2

Microsomes Frozen Two Weeks

Parameter	Conventional method	New method
Glucose-6-phosphatase	0.122 ± 0.010[b]	0.127 ± 0.00[a]
Laurate hydroxylase	0.95 ± 0.022[a]	1.37 ± 0.11[b,c]

[a]A 12% decrease on freezing (same units as Table 1).
[b]No decrease on freezing.
[c]$p < 0.05$, new vs conventional.

Fatty acid compositions of the microsomes prepared by the two methods are summarized in Table 3. The differences cannot be accounted for solely on the basis of differences in peroxidative loss of polyunsaturates and probably reflect differences in the amounts of contamination by other subcellular membranes.

Absorption spectra of zero-time control TBA assays applied to both types of freshly isolated microsomes and read against reagent blanks containing neither sucrose nor mannitol are shown in Figure 2. The background produced by sucrose is obvious, although there is no "peak" at 532 nm as would be produced by MDA (21). In

TABLE 3

Mean Fatty Acid Composition of Microsomal Lipids[a]

Fatty acid[b]	Conventional method	New method
15:0	0.19 ± 0.02	0.21 ± 0.01
16:0	21.0 ± 0.18	22.0 ± 0.91
16:1	1.46 ± 0.10	2.12 ± 0.01[c]
17:0	0.78 ± 0.13	0.66 ± 0.06
17:1	0.19 ± 0.13	0.32 ± 0.04
18:0	21.4 ± 0.23	17.3 ± 0.03[c]
18:1	7.43 ± 0.41	11.5 ± 0.05[c]
19:0	0.11 ± 0.01	0.08 ± 0.01
18:2	14.3 ± 0.08	16.5 ± 0.01[c]
18:3	0.02 ± 0.01	0.10 ± 0.01
20:1	0.63 ± 0.62	0.64 ± 0.06
20:2	0.20 ± 0.10	0.17 ± 0.12
20:3	1.71 ± 0.14	1.15 ± 0.24
20:4	15.8 ± 0.38	13.3 ± 0.05[c]
20:5	1.94 ± 0.16	2.00 ± 0.38
22:5	2.60 ± 0.31	2.91 ± 0.25
22:6	10.32 ± 0.47	9.00 ± 0.12
Total saturates	43.32	40.19
Polyunsaturates	46.91	45.12

[a]Average mol % ± S.D. for three determinations each.
[b]Number of carbon atoms:number of double bonds.
[c]$p < 0.05$.

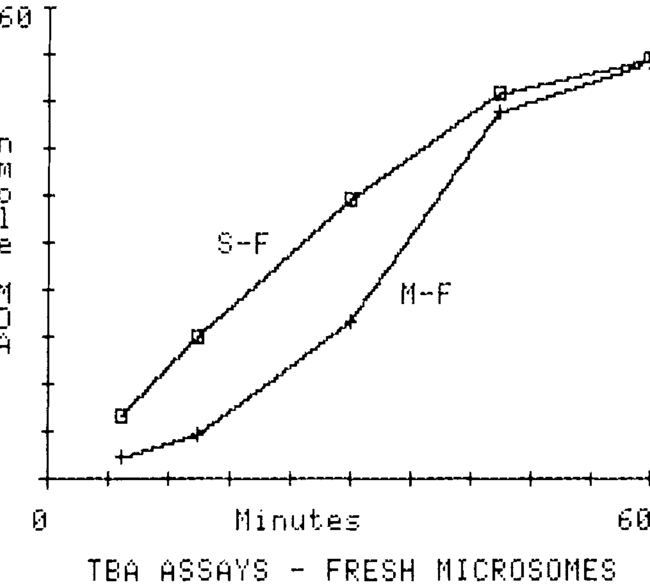

FIG. 3. Time course of production of malondialdehyde by microsomes (1 mg protein/ml) in 25 mM Tris/HCl (pH 7.4) with 100 mM KCl, 330 μM Fe SO_4, 1 mM ADP and 1.85 mM NADPH at 37 C under air on a rotary shaker. S-F, fresh microsomes from sucrose method; M-F, fresh microsomes from mannitol method.

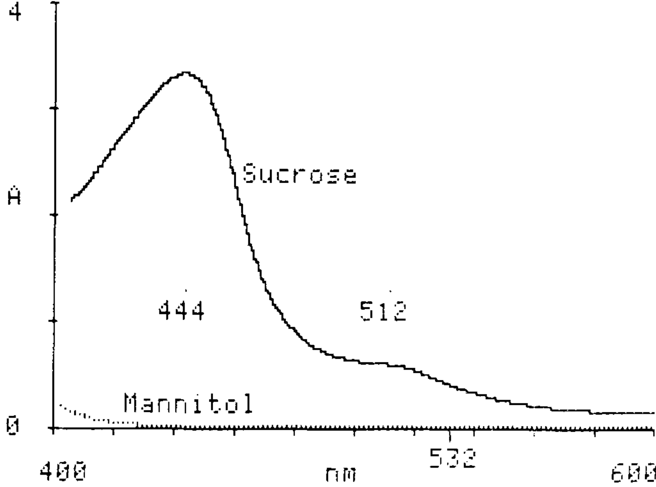

FIG. 2. Visible absorption spectra of TBA reaction products from unincubated microsomes in Tris buffer (zero controls—see text) scanned against procedure blanks lacking microsomes and microsome-suspending media. Upper curve (solid), conventional microsomes (sucrose); lower curve (dotted), microsomes prepared by the proposed method (mannitol).

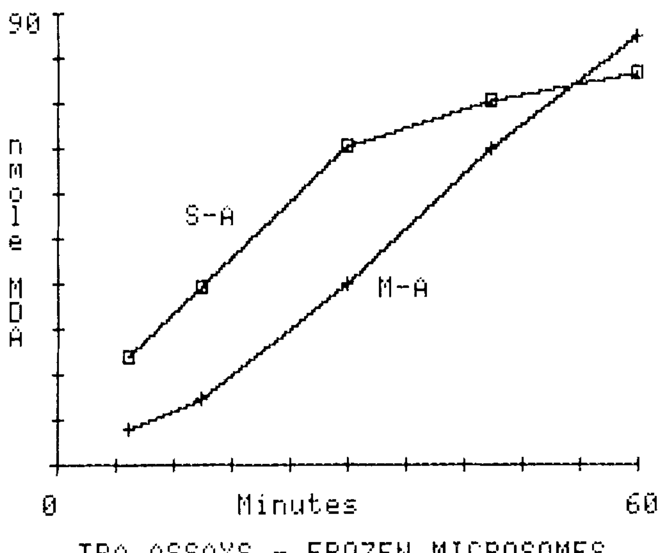

FIG. 4. Time course of production of malondialdehyde by microsomes under the same conditions as Fig. 3 except that the microsomes had been stored at −70 C for two weeks prior to incubation. S-A, "aged" microsomes from sucrose method; M-A, "aged" microsomes from mannitol method.

contrast, microsomes prepared by the new method show no interfering background. Once again, this shows the importance of reading TBA-assay test samples against a zero-time control when sucrose is present (3).

Figure 3 shows the time course of MDA production when microsomes prepared by the two methods were incubated in Tris buffer with NADPH, ferrous sulfate and ADP (3). The colorimetric assay used is relatively specific for MDA rather than its precursors (3). While both types of microsomes eventually produced the same amount of MDA, the "conventional" microsomes gave an initial burst of MDA followed by a parabolic pattern of release. Microsomes prepared by the new method gave a sigmoidal pattern, although there was no true lag. These contrasting patterns were also seen when microsomes that had been stored at −70 C for one month were similarly incubated (Fig. 4).

TABLE 4

Lipid Peroxidation Products in Unincubated Microsomes[a]

Oxidation product	nmol/mg protein	
	Conventional method	New method
Lipid hydroperoxides	10.9 ± 2.34	7.68 ± 1.53
Malondialdehyde (MDA)	0.39 ± 0.061	0.07 ± 0.004[b]
Labile MDA precursors	18.7 ± 0.98	21.8 ± 2.56

[a]Means of triplicate determinations on each of three independent preparations of each type. Microsomes frozen prior to assay, but not incubated.

[b]$p < 0.001$ (two-tailed t-test). Other comparisons not statistically significant.

To determine whether these differences in kinetics were due to differences in levels of preexisting MDA precursors, unincubated (frozen) microsomes were assayed for TBA reactivity both by relatively MDA-specific and nonspecific techniques (3), for the presence of conjugated double bonds in the extracted lipids (17) and for total lipid peroxides/hydroperoxides (18). Solvents used for lipid extraction contained 0.001% butylated hydroxy toluene as antioxidant, and solid EDTA was added to the microsomal suspensions prior to extraction. Results are summarized in Table 4. Microsomes stored in sucrose contained over five times as much preformed MDA as microsomes stored in mannitol and slightly more lipid hydroperoxide. Compounds that could decompose to form MDA were slightly higher in microsomes prepared by the new method. Conjugated double bonds were not readily quantified, since there was no obvious source of microsomal lipid known to be free from such products to use in obtaining a difference spectrum (17). However, the absorbance at 234 nm was not conspicuously different for lipids from the two types of preparations. It was established that even deliberately adding excess TET to the assay mixtures did not interfere with either the TBA assay or the hydroperoxide assay (not shown). Neither sucrose nor mannitol "storage media" had detectable (>0.3 nmol/ml) hydrogen peroxide levels even after storage for 6 mo at 4 C.

A preliminary nonlinear regression analysis (geometric, parabolic, hyperbolic, probit, second-through-fourth order polynomial and inverse transform) of the data in Figures 3 and 4 indicated that while the data for the conventional microsomes could be well fit by a ($y = 1/\text{nmol MDA}$; $x = 1/\text{time}$) transformation, giving correlation coefficients of 0.9966 and 0.9971 for Figures 3 and 4, respectively, the data for microsomes prepared by the new method were better fit by a third-order polynomial (correlation coefficients of 0.9977 and 0.9999, respectively). In the latter case, the second-order term dominated the relationship. The physical correlates of these preliminary observations need to be investigated in the future.

DISCUSSION

The low-speed centrifugation method for preparation of microsomes is adapted from the sucrose-based method of kamath and rubin (19), differing primarily in the composition of the media used. Each mannitol-based solution has an osmotic strength matching that of the corresponding sucrose solution. Mannitol was chosen because (a) unlike sucrose, it does not interfere with the TBA assay or most protein assays; (b) it is a well-known inhibitor of hydroxyl-radical-mediated reactions; and (c) it has been used in the past for subcellular fractionation (although mainly for nonmammalian "microsomes" and mitochondria, e.g., refs. 22, 23).

TET was included in the homogenizing medium because it is an effective chelator of trace metals, has been shown to prevent the formation of hydrogen peroxide during the autooxidation of sulfhydryl compounds (24) and does not interfere with the TBA or lipid hydroperoxide assays (unpublished observations). MOPS buffer was used to prevent the alkaline decomposition of mannitol and because, unlike the more commonly used Tris buffer, it does not partition into the lipid extract and is not itself an antioxidant.

Microsomes can be prepared by the proposed method in about an hour, while several hours are necessary in the conventional method. The rapidly prepared microsomes are at least as pure as the conventional ones, judging by the data in Table 1 and, if used fresh, have a higher level of the peroxidation-sensitive enzyme glucose-6-phosphatase (25). The labile enzyme laurate hydroxylase is preserved better during frozen storage of microsomes prepared by the proposed method than during the storage of conventional microsomes in 0.25 M sucrose.

The enzyme levels in Table 1 for microsomes prepared in mannitol would be consistent with less than 0.1% contamination by mitochondria (26), up to 3.2% lysosomes (27), 1.3% peroxisomes (28) and as much as 5.7% plasma membrane fragments (29). The last estimate is probably high, since microsomes are themselves thought to contain some 5′-nucleotidase (30).

Incubation of the microsomes in the presence of NADPH, ferrous sulfate and ADP gives, after 1 hr, about the same amount of MDA for both types of preparations. However, the time course of the process is quite different in the two. Since MDA production occurs at the end of a sequence of steps involving the incorporation of oxygen into a fatty acid chain at at least two points, one would expect the rate of MDA release to increase as intermediates in the process accumulate. This is what is observed for microsomes prepared by the proposed method. In contrast, MDA release from conventional microsomes was initially very fast, slowing continuously throughout the course of the incubation.

The differences in MDA release patterns cannot be explained by any difference in the content of polyunsaturated fatty acids, since that was similar in both. The content of preformed oxidation products, except for MDA itself, was similar in both. The total amount of MDA released in one hour was only about 5% of the amount of NADPH provided, and the microsomes prepared by the new method were still releasing MDA at close to their maximum rate. Even had the microsomal suspending medium contained as much TET as the homogenizing medium, there would have been a 33-fold excess of ferrous sulfate present during the incubation. Since the suspending medium did not contain TET, the excess of ferrous ion must have been much greater than 33-fold.

Among the possible causes for the difference in MDA-release kinetics are (a) the microsomes prepared by the new method would have had weakly bound metal ions effectively "extracted" by the TET in the homogenizing medium, while those ions would have remained in the conventional microsomes; (b) the conventional microsomes may have contained peroxidation intermediates differing in kind, if not in amount, from those present in the other preparation; (c) the physical difference in the preparatons, with many free ribosomes in the conventional prep and only adsorbed ribosomes in the mannitol-based prep may have influenced the transfer of products. The fact that the cause for the difference in kinetics cannot be given an obvious explanation supports the suggestion that microsomes prepared by the proposed method may be useful in studies of the mechanistic aspect of lipid peroxidation.

A second advantage of the proposed method is clear from Figure 2. Avoiding sucrose eliminates the correctable but high background in the TBA assay, making it possible to more accurately measure low levels of peroxidation products. Even trace levels of MDA may be considered significant, since the mitochondrial contamination of these preparations is so low that MDA metabolism will be insignificant.

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COMMUNICATIONS

The Differential Effect of Polymyxin B_1 on Guinea Pig Lung Mitochondrial and Microsomal Glycerophosphate Acyltransferase

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The activities of guinea pig lung mitochondrial and microsomal glycerophosphate acyltransferase differed in sensitivity to polymyxin B_1. At an antibiotic concentration of 1 mg/ml, the mitochondrial enzyme activity was stimulated twofold, but the microsomal enzyme activity was completely inhibited. Furthermore, the mitochondrial enzyme activity was stimulated by polymyxin B_1 without the addition of exogenous acyl-CoA. Additional experiments ruled out the possibility of polymyxin B_1 acting as a substrate for the mitochondrial acyltransferase. These results suggest either that the polymyxin B_1 sensitivity of mitochondrial and microsomal glycerophosphate acyltransferase is different or that their accessibility to substrates is different because the two isoenzymes are located differently in the different phospholipid microenvironment of the membranes.
Lipids 22, 757-759 (1987).

We have previously demonstrated a biomodal distribution of glycerophosphate acyltransferase activity in guinea pig lung and suggested that this enzyme might play an important role in lung development (1). Recent studies with liver and lung have indicated that a portion of this enzyme is exposed on the cytosolic side of the endoplasmic reticulum (2,3), whereas in mitochondria the enzyme spans the transverse plane of the outer membrane (4-8). Our results (1) indicated that lung mitochondrial and microsomal glycerophosphate acyltransferase have different functional characteristics. For example, while the microsomal enzyme activity was inhibited by N-ethylmaleimide, trypsin and acetone, the mitochondrial enzyme activity was either unaffected or stimulated. The exact mechanisms for this differential response are unclear. However, it is possible that particular locations of the enzymes in the respective membranes in relation to exposure to cytosolic environment may be responsible for this phenomenon. To substantiate this hypothesis, we searched for a probe with a strong binding affinity toward biological membranes. It is expected that such a binding would alter the activity of an enzyme depending on whether it is located in the outer or inner surface of the membrane. For this purpose, we used polymyxin B_1, a cyclic polypeptide containing one 8- to 9-carbon fatty acid residue, as a probe. Polymyxin B_1 is known to have a strong bactericidal activity due to its binding to the plasma membrane. It causes swelling of the outer leaf of the outer membrane and thereby disturbs the normal organization of the membrane and alters its permeability characteristics (9).

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MATERIALS AND METHODS

Male guinea pigs (Hartley strain) of 300-g body weight were used. Their lungs were removed and washed with cold saline and with 0.25 M sucrose and were hand-homogenized in 0.25 M sucrose/0.01 M Tris, pH 7.4 (5 ml/g lung) with a Potter Elvehjem homogenizer. The homogenate was centrifuged at 1,000 × g for 10 min. The supernatant fluid was centrifuged at 20,000 × g for 20 min. The pellet was resuspended in a sucrose-Tris buffer and resedimented at 6,500 × g. The sediment (6,500 × g) was washed three times with sucrose-Tris buffer. It was finally resuspended in the same medium and used as mitochondrial preparation. The 20,000 × g supernatant fluid was centrifuged at 105,000 × g for 60 min to sediment the microsomes. The microsomal pellets were washed two times and resuspended in sucrose-Tris buffer. Protein was estimated according to Lowry et al. (10). Mitochondrial and microsomal cross-contaminations were evaluated by measuring marker enzymes, such as NADPH cytochrome c reductase and succinic dehydrogenase (11), as well as N-ethylmaleimide sensitivity of the subcellular glycerophosphate acyltransferase as described previously (1). Glycerophosphate acyltransferase assays were performed in a total volume of 0.5 ml as described in previous studies (1). All assays contained an optimal concentration of palmitoyl- or oleoyl-CoA (72 μM for mitochondria and 144 μM for microsomes), 1.5 mM *sn*-[2-^{3}H]glycerol 3-phosphate with a specific radioactivity of 10×10^3 cpm per nmol and ca. 0.1 mg subcellular protein. Polymyxin B_1 was purchased from Sigma Chemical Co. (St. Louis, MO) and added at 0.1, 0.2, 0.4, 1.0 and 2.0 mg/ml to the reaction mixture. The reaction was initiated by the addition of a subcellular fraction and terminated after 3 min by the addition of 1 ml of 1-butanol. An aliquot (0.7 ml) of the washed butanol extract was taken in a scintillation vial, evaporated under nitrogen and mixed with aquasol (5 ml) and counted in a Beckman LS-355 scintillation counter.

RESULTS AND DISCUSSION

The mitochondrial preparation had high succinic dehydrogenase (23.88 ± 8.56 nmol/min/mg; 5 determinations) and low NADPH cytochrome c reductase activity (12.37 ± 1.10 nmol/min/mg protein, 5 determinations). The microsomal preparation, by contrast, had high NADPH cytochrome c reductase (47.37 ± 1.10 nmol cytochrome c reduced/min/mg) and little succinic dehydrogenase activity (2.65 ± 1.55 nmol formazan/min/mg). Furthermore, at NEM concentration of 8.0 mM, the microsomal glycerophosphate acyltransferase activity was inhibited by 90%, whereas the mitochondrial activity was completely resistant. Thus, the mitochondrial and microsomal preparations were reasonably pure.

As shown in Figure 1, the treatment with polymyxin B_1 provides another method by which lung mitochondrial and microsomal glycerophosphate acyltransferase could be distinguished. In the presence of palmitoyl-CoA as the donor, polymyxin B_1 (1.0 mg/ml) caused nearly 150% stimulation of the mitochondrial enzyme activity while almost completely inhibiting the corresponding microsomal enzyme. Qualitatively similar differential effect of polymyxin B_1 was observed when oleoyl-CoA was used instead of palmitoyl-CoA as the acyl donor. The inhibitory action of polymyxin B_1 on microsomal enzyme was maximal within 1 min of incubation. Polymyxin B_1 has also been shown to have similar differential effects on mitochondrial and microsomal glycerophosphate acyltransferase in rat liver (5).

Thin layer chromatographic analysis of acylation products (lysophosphatidic acid and phosphatidic acid) using a solvent system, diisobutyl ketone/acetic acid/water (40:25:5, v/v/v), indicated that the polymyxin B_1 addition to the incubation medium of mitochondrial fraction did not alter the percent composition of the mono- (47.6%) and diacylated (52.4%) products. It should be noted that the condition used for the assay of glycerol 3-phosphate acylation were such that there was linearity with time and with the amount of protein. Because the ratio of the amount of lysophosphatidic acid and phosphatidic acid did not change in the presence of polymyxin B_1, the increased acylation of glycerol 3-phosphate by polymyxin B_1 is probably due to the stimulation of both acylation steps.

The difference in polymyxin B_1 sensitivity between the mitochondrial and microsomal glycerophosphate acyltransferase might be due to differences in the phospholipid microenvironment of the membranes or the orientation of the enzymes within the respective membrane. It is interesting to note that in the absence of an exogeneously added acyl donor, the endogenous mitochondrial enzyme activity (0.4 nmol/min/mg) was stimulated approximately 17-fold by polymyxin B_1 (1 mg/ml), whereas the endogenous microsomal enzyme activity remained unaffected by the antibiotic (Table 1). This observation indicates that polymyxin B_1 might partly act as a substrate for the mitochondrial enzyme, especially because the stimulation of the mitochondrial acyltransferase due to the addition of polymyxin B_1 (17-fold) was even more than that due to the addition of palmityl-CoA (threefold). Alternatively, the antibiotic interacts with the phospholipid bilayer of the mitochondrial outer membrane and perturbs the phospholipid microenvironment of the acyltransferase.

In order to investigate whether polymyxin B_1 may partly act as a substrate for the mitochondrial enzyme, the mitochondrial fraction was incubated with 72 μM-[palmitoyl-1-^{14}C]palmitoyl CoA (40 mCi/mmol), and varied concentrations of polymyxin B_1 (0.1, 0.2, 0.4, 0.6, 1.0 and 2.0 mg/ml) and glycerophosphate acyltransferase activities were determined. Polymyxin B_1 addition to the incubation medium caused a stimulation on the incorporation of radiolabeled substrate into phospholipids (Table 2). This observation suggests that polymyxin B_1 is not used as a substrate, but rather stimulates the accessibility of the mitochondrial glycerophosphate acyltransferase to exogenous palmitoyl CoA or to endogenous acyl donors.

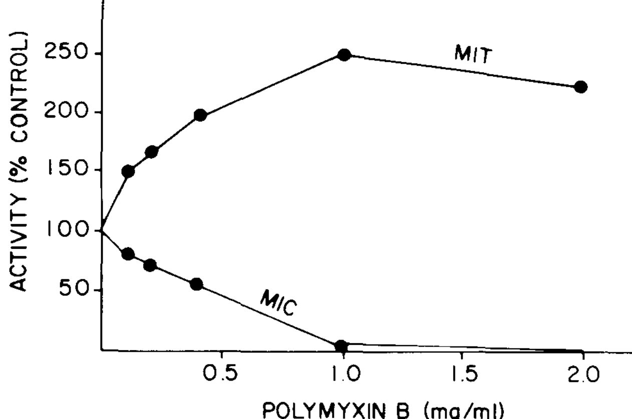

FIG. 1. Differential action of polymyxin B_1 on mitochondrial and microsomal glycerophosphate acyltransferase. The reaction was initiated by addition of the subcellular fraction to the incubation mixture. Palmitoyl-CoA was used as the acyl donor at 72 μM concentration for the mitochondria and 144 μM for the microsomes. Polymyxin B_1 was added as indicated. The specific activities of the subcellular fractions in the absence of polymyxin B_1 were 1.3 ± 0.1 and 2.9 ± 0.3 nmol/min/mg protein for mitochondria and microsomes, respectively.

TABLE 1

Effect of Polymyxin B_1 on Guinea Pig Lung Mitochondrial and Microsomal Glycerophosphate Acyltransferase Activity

	Enzyme activity (nmol glycerol 3-phosphate incorporated/min/mg protein)	
Additions to incubation medium	Mitochondria	Microsomes
None	0.40 ± 0.01	0.60 ± 0.01
Palmitoyl CoA	1.31 ± 0.10	2.91 ± 0.31
Polymyxin B_1	6.82 ± 0.82	0.71 ± 0.01
Palmitoyl CoA + polymyxin B_1	7.61 ± 0.51	0.62 ± 0.02

Assay conditions for glycerophosphate acyltransferase are described under Materials and Methods. Palmitoyl-CoA at concentrations of 72 μM for mitochondria and 144 μM for microsomes was used as the acyl donor. Polymyxin B_1 at a concentration of 1 mg/ml was used for both mitochondrial and microsomal fractions. Each value is the mean ± S.D. of 5 experiments.

TABLE 2

Effect of Polymyxin B_1 on Guinea Pig Lung Mitochondrial and Microsomal Glycerophosphate Acyltransferase Activity

Addition of polymyxin to incubation medium (mg/ml)	Enzyme activity (nmol palmitoyl CoA incorporated/min/mg protein)
None	1.8 ± 0.3
0.1	2.9 ± 0.3
0.2	4.6 ± 0.6
0.4	6.2 ± 0.4
0.6	7.3 ± 0.6
1.0	8.2 ± 0.7
2.0	6.9 ± 0.5

Assay conditions for glycerophosphate acyltransferase are described under Materials and Methods. [Palmitoyl-1-^{14}C]palmitoyl CoA (40 mCi/mmol) at a concentration of the 72 μM was used as substrate. Each value is the mean ± S.D. of 3 experiments.

As glycerophosphate acyltransferase is a membrane-bound enzyme, its activity is regulated by different phospholipid species (12). If polymyxin B_1 binds with the phospholipids of the outer side of the mitochondrial outer membrane, these phospholipid molecules, which take part in modulating the mitochondrial glycerophosphate acyltransferase activity, will be affected. We have recently shown that in lung (6), mitochondrial enzyme is stimulated twofold in the presence of a proteolytic enzyme such as trypsin. As the mitochondrial outer membrane is impermeable to trypsin, this stimulation could be regualted from the cytosolic side of the mitochondrial outer membrane. Indeed, these results and results with liver mitochondria (8) indicate that the mitochondrial glycerophosphate acyltransferase is a transmembrane protein. Currently, we are studying the effects of polymyxin B_1 on glycerol phosphate acylation and monoamine oxidase activity in lung mitochondrial outer membrane preparations.

In an isoionic medium, the mitochondrial enzyme might not be exposed to the aqueous environment outside the mitochondria (5), but a portion of the enzyme could still be present imbedded in the phospholipid layer of the outer side of the mitochondrial outer membrane. It is this portion that is involved in the manifestation of the stimulatory action of polymxin B_1 on mitochondrial glycerophosphate acyltransferase.

ACKNOWLEDGMENTS

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A Facile Synthesis and Carbon-13 Nuclear Magnetic Resonance Spectral Properties of 7-Ketocholesteryl Benzoate

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This paper presents a modified method of the selective allylic oxidation of cholesteryl benzoate. Pyridinium chlorochromate, in refluxing benzene, has been found to be an effective and convenient reagent for the efficient oxidation of cholesteryl benzoate to 7-ketocholesteryl benzoate in high yield. Also included herein are the carbon-13 nuclear magnetic resonance spectral properties of 7-ketocholesteryl benzoate and cholesteryl benzoate.
Lipids 22, 760–763 (1987).

Among the most frequently encountered oxidation products of cholesterol are those with an oxygen function at C-7 (1,2). Many of these oxidized derivatives may be formed enzymically from cholesterol or may be the result of autoxidation. These compounds have been found in animal tissues and foodstuffs (2) and are known to be inhibitors of mammalian sterol biosynthesis (3–6) and cell replication (7–9).

As a result of our continuing studies on sterol biosynthesis, we have developed a simplified method for the synthesis of 7-ketocholesteryl benzoate, 2, a key intermediate in the synthesis of 7-oxygenated sterols. The method reported herein is applicable to both small- and large-scale preparations and represents an improvement in convenience and yield over previously reported procedures.

Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectral studies can provide far more information concerning the structure of steroids than proton (^{1}H) NMR studies, since most of the carbons exhibit separately resolved signals that may be assigned to specific carbon atoms with a high degree of confidence (10). As an extension of our synthetic studies, we have completed the unambiguous ^{13}C NMR assignments for carbon atoms in 7-ketocholesteryl benzoate, 2, and cholesteryl benzoate, 1.

1 R_1 = H, R_2 = H   2 R_1 = R_2 = O

SCHEME 1. Steroid structures of *1* and *2* and ring numbering system.

EXPERIMENTAL METHODS

General methods. Melting points were determined with an Electrothermal capillary apparatus and are uncorrected. Infrared (IR) spectra (KBr pellet) were recorded on a Perkin-Elmer Model 580 spectrometer. ^{1}H NMR and ^{13}C NMR spectra (deuterated chloroform solvent) were obtained with a Bruker AM-400 spectrometer operating at a frequency of 400.179 MHz for ^{1}H and 100.619 MHz for ^{13}C using tetramethylsilane as an internal standard. Proton spectra were obtained using 16K data points and a sweep width of 10 ppm, while parameters for carbon spectra were 32K and 250 ppm, respectively. Distortionless enhancement polarization transfer (DEPT) experiments (11,12) were conducted at $\theta = 135°$, which corresponds to a pulse width of 21.4 sec. Carbon-hydrogen (C–H) correlation (13,14) was conducted using 2K data points in the F2 dimension and 512 data points in the F1 dimension. The F1 data was zero-filled to 1K, and exponential line broadening (2 Hz in F2 and 0.25 Hz in F1) was included before Fourier transformation. Proton chemical shifts for the C-18 and C-19 angular methyl resonances were calculated by the method of Zurcher (15). Mass spectral (MS) analysis was conducted on a VG-7070E magnetic sector mass spectrometer at 70 eV (source temperature was 150 C) using a solid probe, and the results are presented in terms of relative intensity (percentage of the base peak) along with probable mode of origin. Gas liquid chromatographic (GLC) analysis was performed on a Hewlett-Packard (HP) gas chromatograph (5710 A) equipped with a 25-M glass capillary column coated with DB-5 and a flame ionization detector. Thin layer chromatography (TLC) was carried out on plates of Silica Gel G (Analtech, Newark, DE) with the components visualized after being sprayed with molybdic acid (16). Solvent systems for TLC analysis were SS-1, toluene, SS-2, 10% ether in hexane and SS-3, 10% ethyl acetate in hexane. Column chromatography employed silica gel (60–200 mesh) on columns that were 100 cm × 1.5 cm. Cholesteryl benzoate was prepared in 89% yield by treatment of purified commercial cholesterol (98% pure by GLC analysis) with benzoyl chloride in pyridine (mp 148.5–150 C, lit. [17] mp 147 C). Commercial cholesterol (Sigma Chemical Co., St. Louis, MO) was purified by multiple recrystallizations from acetone-water. A sample of authentic 7-ketocholesteryl benzoate was prepared by the procedure of Salmond et al. (18). Pyridinium chlorochromate (PCC) was obtained from the Aldrich Chemical Co. (Milwaukee, WI).

General oxidation procedures. Cholesteryl benzoate (4.91 g, 0.01 mol) was dissolved in benzene (250 ml) containing molecular sieves (0.5 g, type 3A). Pyridinium chlorochromate (PCC, 64.67 g, 0.30 mol) was added, and the stirred reaction mixture was gently refluxed under

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Abbreviations: ^{13}C NMR, carbon-13 nuclear magnetic resonance; ^{1}H NMR, proton nuclear magnetic resonance; IR, infrared; DEPT, distortionless enhancement polarization transfer; MS, mass spectra; GLC, gas liquid chromatography; HP, Hewlett-Packard; TLC, thin layer chromatography; PCC, pyridinium chlorochromate; DMF, N,N-dimethylformamide; DMSO, dimethylsulfoxide.

a nitrogen atmosphere for 24 hr. The benzene solution was decanted, and the remaining contents of the reaction flask were washed several times with ether. The combined extracts were washed with a saturated NaCl solution, dried over anhydrous magnesium sulfate and evaporated to dryness under reduced pressure. TLC analysis (SS-1) indicated the presence of a single component of ca. 95% purity. The residue was subjected to column chromatography using a solvent gradient of ether in toluene. The purified material was recrystallized from acetone-water to yield 7-ketocholesteryl benzoate (4.39 g, 87%): mp 158–159.5 C, lit. (17) 159 C; IR ν_{max} 1735, 1685, 1670, 1245, 1039 cm^{-1}; ^1H NMR: 0.69 (S, 3H, C−18−CH_3), 1.25 (S, 3H, C−19−CH_3), 4.93 (m, 1H, C−3−H), 5.73 (m, 1H, C−6−H), 7.73 (m, 5H, benzoate); ^{13}C NMR: (Table 1); MS, m/e (rel int): 504 (M, 4%), 382 (M-benzoic acid, 7%), 367 (M−CH_3−benzoic acid, 2.8%), 121 (benzoyloxy, 5%), 105 (benzoyl, 100%). GLC analysis indicated a single major component of 98% purity, and TLC analysis indicated a single component in three solvent systems (SS-1, SS-2 and SS-3). Both analyses showed identical chromatographic mobility with an authentic compound.

RESULTS AND DISCUSSION

The allylic oxidation of Δ^5-steroids was initially accomplished by chromic acid reagents with only limited success (19). For example, the allylic oxidation of **1** with sodium chromate in acetic acid/acetic anhydride gave **2** in an optimum yield of 38% (18).

Synthetically useful changes in the properties and reactivity of chromium (VI) reagents have been brought about by the formation of amine complexes. The Collins reagent is formed by the complexation of chromium trioxide with pyridine (20,21). Using this reagent, the allylic oxidation of **1** gave a 68% yield of **2** (17), and in a related study, using very anhydrous conditions, cholesteryl acetate was oxidized to 7-ketocholesteryl acetate in 72% yield (22). Similar complexes have been formed using chromium trioxide and pyrazole (23), 3,5-dimethylpyrazole (18) and benzotriazole (24) and have been shown to oxidize **1** to **2** in 70–76% yields. These reactions require the preparation of the reagent complex before each reaction.

Commercially available PCC has recently become widely used in organic synthesis for the oxidation of

TABLE 1

Carbon-13 Chemical Shifts for 7-Ketocholesteryl Benzoate (*2*) and Cholesteryl Benzoate (*1*)[a]

Carbon atom	2	1	Difference (ppm)
1	36.0	37.1	−1.1
2	27.5	27.9	−0.4
3	72.7	74.6	−1.9
4	37.8	38.3	−0.5
5	163.6	139.7	23.9
6	126.7	122.7	4.0
7	201.5	32.0	169.5
8	45.4	32.0	13.4
9	50.0	50.1	−0.1
10	38.3	36.7	1.6
11	21.2	21.1	0.1
12	28.5	28.3	0.2
13	43.1	42.4	0.7
14	49.8	57.7	−7.9
15	26.3	24.3	2.0
16	38.7	39.8	−1.1
17	54.8	56.2	−1.4
18	11.9	11.9	0.0
19	17.3	19.4	−3.5
20	35.7	35.8	−0.1
21	18.9	18.8	0.1
22	36.2	36.2	0.0
23	23.8	23.9	−0.1
24	39.5	39.6	−0.1
25	28.0	28.0	0.0
26	22.5	22.6	−0.1
27	22.8	22.8	0.0
C_{ipso}	130.2	130.9	−0.7
C_{ortho}	129.5	129.5	0.0
C_{meta}	128.3	128.2	0.1
C_{para}	132.9	132.6	0.3
Ester[b]	165.6	165.9	−0.3

[a]In ppm downfield from tetramethyl silane.

[b]Benzoate ester carbonyl group.

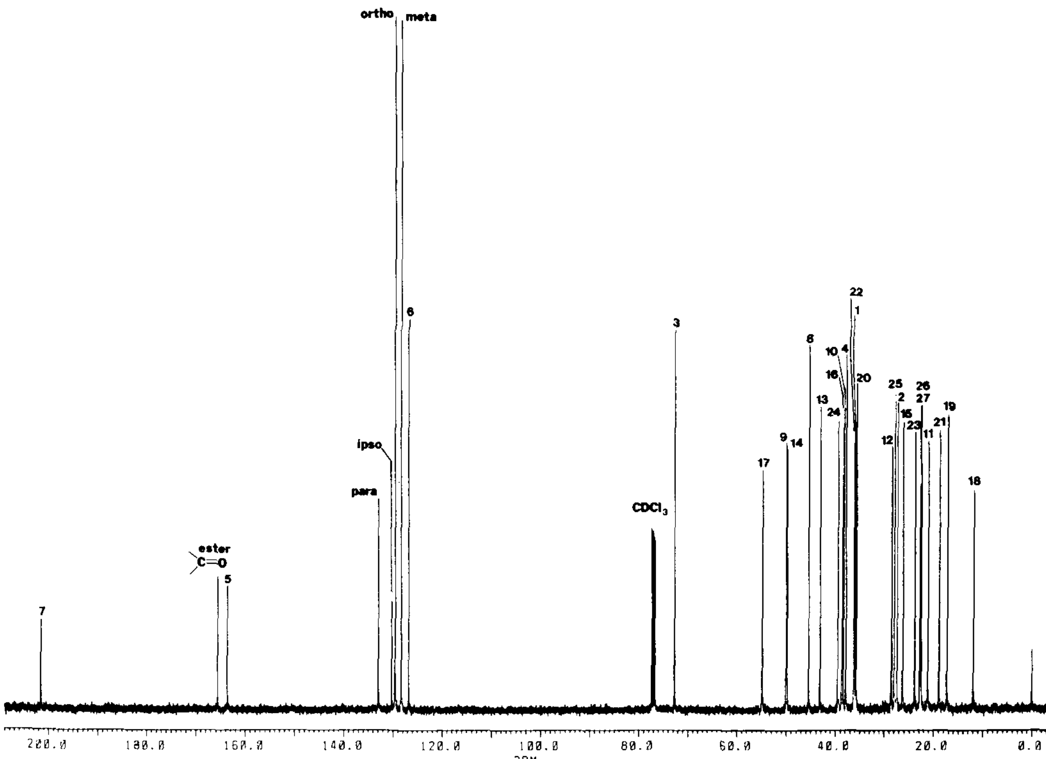

FIG. 1. A decoupled carbon-13 NMR (100.619 MHz) spectrum of 7-ketocholesteryl benzoate (*2*) with carbon assignments. Resonances are downfield from tetramethylsilane.

primary and secondary alcohols to carbonyl compounds (25). This reagent, in methylene chloride containing pyridine (26), other aromatic amines (27), pyrazole (28), 3,5-dimethylpyrazole (29) and benzotriazole (24), was reported to effect the selective oxidation of the allylic hydroxyl function of a number of steroidal alcohols. At room temperature, PCC in methylene chloride was found to be an ineffective reagent for allylic oxidation (18). In contrast to these latter results, we have achieved moderate success using PCC in refluxing methylene chloride for allylic and benzylic oxidations (30). PCC, in dimethylsulfoxide, has also been used for the oxidation of β-ionone to the corresponding diketone (31).

In the present study, we have found that PCC, in refluxing benzene, can affect the high yield (87%) oxidation of 2 to 1. This conversion was accomplished with a 1:30 ratio of reagent (PCC) when 1–10 g of 2 were oxidized. Oxidations of 2 conducted on quantities of less than 1 g were successfully performed using smaller quantities of reagent (1:25) with similar yields, thus demonstrating the ability of the described method to be useful for both large- and small-scale preparations. This efficient procedure represents a significant improvement in both yield and convenience compared to other reported methods for the allylic oxidation of 1 and 2.

Using the reaction conditions described herein, additional studies were conducted using other solvents with 1 and PCC. Under these conditions, refluxing acetone, pyridine, N,N-dimethylformamide (DMF) and dimethylsulfoxide (DMSO) at 100 C yielded 2 in 2%, 0%, 18% and 77% yield, respectively. In a previous study, we found the use of refluxing methylene chloride as solvent yielded 54% of 2 from 1 (30).

As an extension of our synthetic studies, we have completed the unambiguous ^{13}C NMR assignments for carbon atoms in 7-ketocholesteryl benzoate, 2, and cholesteryl benzoate, 1 (Table 1, Fig. 1). The previously reported ^{13}C spectrum of cholesteryl acetate (10,32) was used to assign resonances due to carbons 16–18 and 20–27. Carbons of the benzoate group were assigned using standard spectra of benzoate esters (33). A DEPT (11,12) experiment separated the remaining carbon resonances into two groups, secondary (down) and primary or tertiary (up). An additional C—H correlation experiment (13,14) provided data to unambiguously assign the peaks at 45.4, 49.8 and 50.0 ppm to carbons 8, 9 and 14, respectively.

Evaluation of the C—H correlation spectrum indicated that the carbon peaks at 45.4, 49.8 and 50.0 correlated with proton (^{1}H) multiplets at 2.25, 1.55 and 1.35 ppm, respectively.

The assignment of 45.4 ppm at carbon 8 was straightforward, since the proton multiplet at 2.25 ppm is adjacent to the carbonyl function at carbon 7 and is characteristically shifted to a downfield position (deshielded) (34). The assignment of 49.8 ppm as carbon 14 and 50.0 as carbon 9 was based on the observation that the C-14 proton is closer to the ketone function at carbon 7 and is, therefore, more deshielded than the C-8 proton (1.55 ppm vs 1.35 ppm). It is also interesting to note (based on the C—H correlation spectrum) that the protons on C-15 appear at 1.22 and 2.42 ppm. The proton shifted downfield to 2.42 ppm is the proton that, upon

inspection of models, is seen to be quite close to the carbonyl at carbon 7.

From the results presented in Table 1, the introduction of the ketone function at carbon 7, 2, causes several major changes, notably at carbons 7, 8 and 14 and at the olefinic carbons 5 and 6. By the use of the DEPT and C—H correlation techniques, the assignments of the carbons in 1 and 2 were conveniently made in a timely manner. The results obtained were in favorable agreement with the salient carbon assignments for cholesteryl acetate and 7-ketocholesteryl acetate made using earlier ^{13}C NMR techniques (10,32).

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Immunocytochemical Localization of Lingual Lipase in Serous Cells of the Developing Rat Tongue

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The ontogeny of the rat lingual serous and mucous glands was explored by light and electron immune microscopy using the peroxidase-antiperoxidase and streptavidin-gold techniques. Tissues from fetal and neonatal rats from day 18 of gestation through 4 wk after birth were fixed and embedded in paraffin or Epon for light and transmission electron microscopy, respectively. Electron microscopy revealed that the only cells containing lingual lipase were the developing serous cells; secretory granules containing lingual lipase of varying degrees of maturity were seen. Mucous cells did not show immunospecific staining in rats of any age. The neonatal "mixed" lingual glands secrete lingual lipase from serous components immediately after birth to aid in fat digestion.
Lipids 22, 764-766 (1987).

Lingual lipase is an important enzyme in gastric fat digestion in man and other animals (1-5). We have recently demonstrated by immunocytochemical methods using a polyclonal monospecific antibody to lingual lipase that the enzyme is found primarily in the serous von Ebner glands located beneath the circumvallate papilla of the tongue in adult rats (5). Serous demilune cells of the lingual mucous glands also contain lingual lipase. However, the mucous cells of adult rats do not contain this enzyme (5). The aim of this study was to explore the ontogeny of rat lingual lipase, using immunocytochemical techniques to determine at what stage of development and in what specific cell type this enzyme first appears.

MATERIALS AND METHODS

Lingual lipase was purified from adult male Sprague Dawley rats, and polyclonal antibodies were raised in female New Zealand rabbits as previously described (5).

Chemicals. Octylglucoside was purchased from Sigma Chemical Co. (St. Louis, MO). Sephacryl S-200, polybuffer 74 and PBE 94 were products of Pharmacia Fine Chemicals (Piscataway, NJ). Buffers were composed of ACS-grade chemicals (Fisher Scientific Co., Pittsburgh, PA). Other chemicals were of the highest purity available.

Immunocytochemical localization of rat lingual lipase light microscopy. The method for light immunomicroscopy was as previously described (5) except that tissues were immersed in 10% alcoholic formalin/95% alcohol (1:9, v/v) and fixed overnight. The specimens were processed on an Autotechnicon and embedded in Tissue Prep (paraffin and synthetic polymer, Fisher Scientific). Tissue from day-1 postpartum rats was obtained prior to the initiation of suckling.

Sections were cut at 4-5 μm, hydrated through xylene and 95% alcohol to water. Endogenous tissue peroxidases were depleted with 1% hydrogen peroxide in methanol for 30 min. Immunospecific staining was not observed when sections were stained with pre-immune serum or second antibody (goat antirabbit IgG) in the absence of primary antibody (rabbit antilipase).

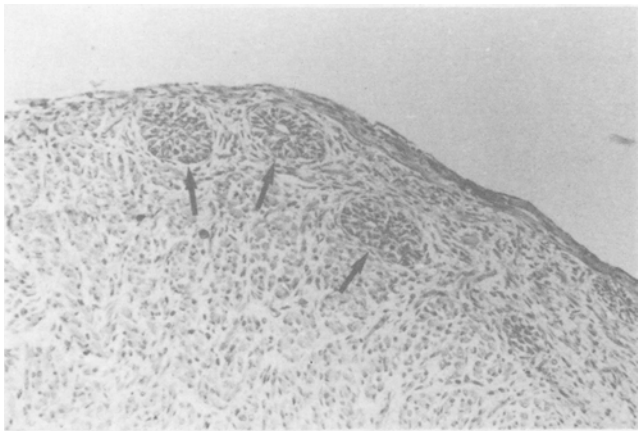

FIG. 1. Immature glands (arrows), day 18 of gestation. There is no immunospecific staining for lingual lipase at this time. (40×, hematoxylin and eosin).

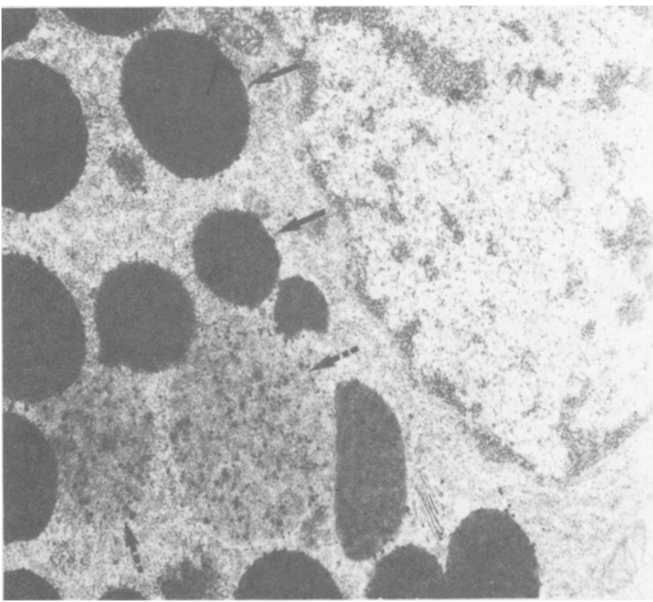

FIG. 2. Day 1 after birth. Gold particles are restricted to the serous granular cells. There is gold found in association with mature (compact; solid arrows) and immature (light fuzzy; dashed arrows) secretory granules. The granules exhibit varying density. (27,000×, streptavidin-gold).

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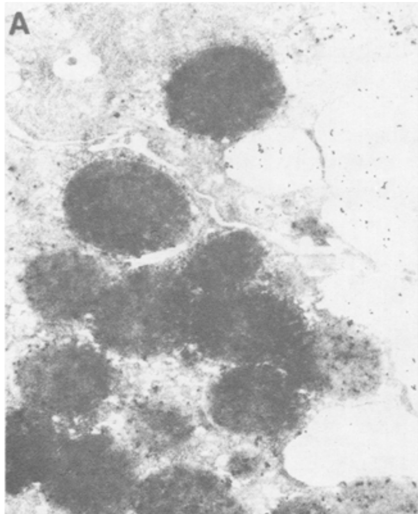

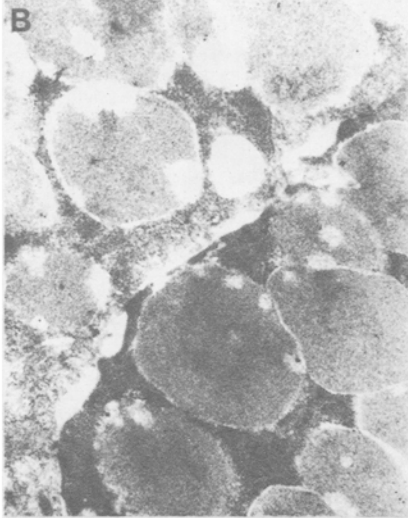

FIG. 3. Day 1 after birth. (a) Serous cells demonstrate gold in association with all granules. Exocytosis of lingual lipase is evident by the presence of gold in the ductular lumen (27,000×, streptavidin-gold). (b) Mucous cells from "mixed" glands. Note the absence of specific immunostaining for lingual lipase by the streptavidin-gold technique (27,000 ×, streptavidin-gold).

Additional sectons were stained for mucin by the mucicarmine method and for glycoprotein by the periodic acid-Schiff (PAS) method (6).

Electron microscopy. The method for electron microscopy was as previously described (5) except that sections of rat tongue containing lingual serous and mucous glands were immersed in 1% glutaraldehyde at 4 C for only 1 hr, followed by washing in 0.2 M phosphate buffer with 10% sucrose for 48 hr. Tissues were postfixed, dehydrated, embedded and stained for electron microscopy using the biotin-streptavidin technique (ABC) previously described (5). Immunospecific staining was not seen with pre-immune serum, second antibody or streptavidin-gold in the absence of rabbit antilipase (first antibody).

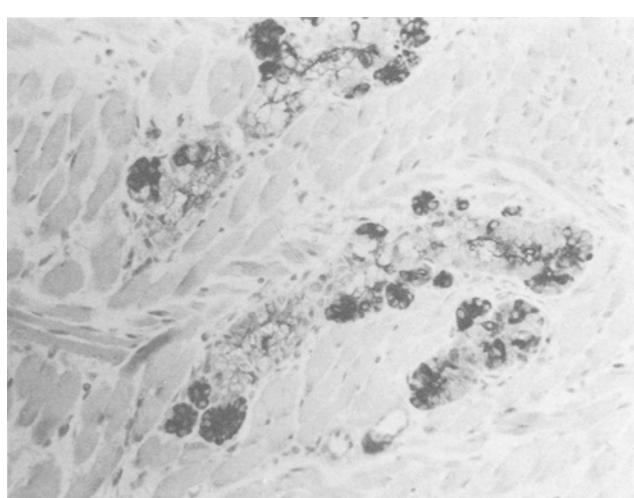

FIG. 4. Day 3 after birth. "Mixed" serous and mucous glands. Note the intense immunospecific staining for lingual lipase in the serous cells (100×, peroxidase-diaminobenzidine).

RESULTS

Primitive cellular cords appeared in the subepithelial stroma of the dorsum of the posterior tongue for the first time on the 18th day of gestation (Fig. 1). The cords showed no stainable mucin, glycogen or lingual lipase. In addition, lingual lipase was not detectable on day 19. Some primitive glands between days 20 and 21 appeared to stain positively for the presence of mucin and lingual lipase. On day 22 of gestation (prepartum), all glands contained stainable lingual lipase (peroxidase-antiperoxidase method, PAP), mucin and glycogen and were easily recognized on light microscopy. By the first postpartum day, three cell types were identified by electron microscopy in these "mixed" glands: (a) undifferentiated cells without cytoplasmic specialization; (b) serous cells with dark, apical cytoplasmic granules; and (c) mucous cells with characteristic mucous granules. The serous cells contained cytoplasmic secretory granules of varying degrees of maturity (Fig. 2). Immune electron microscopy showed lingual lipase in all serous granules, and there was lipase detectable in the glandular lumens (Fig. 3a). Neither the undifferentiated nor the mucous cells had evidence of lingual lipase (Fig. 3b). By the third postpartum day, there were fewer undifferentiated cells in the mixed glands, and mucous cells were the predominant cell type. The serous cells of the mixed glands contained larger granules and there were more gold particles per granule than in the day 1 specimens. At this stage, also, exclusively serous acini accompanied the mixed glands, i.e., reminiscent of the adult rat tongue (5). The serous glands contained glycogen, but did not stain positively for mucin. Both the serous and mixed glands demonstrated stainable lipase by light microscopy (Fig. 4). However, by the

fourteenth postpartum day, the mixed glands had developed a predominantly mucous adult appearance with only occasional lipase-containing demilune cell. The serous glands also then showed the branching ducts and multiplicity of acini of the adult rat tongue.

DISCUSSION

We have shown that, in the adult rat, lingual lipase appears to be confined to the serous von Ebner glands and demilune cells of the lingual mucous glands (5). Other investigators have shown that there is abundant lipolytic activity present in the tongue of the newborn rat (1-4) and have histologically studied the ontogeny of the lingual mucous and serous glands (1). We have demonstrated that rat lingual lipase is immunocytochemically clearly detectable in all mixed glands as early as the 20th to 22nd gestational days. It is difficult, however, using light microscopy to identify the precise cell that contains the enzyme because the entire gland stains for mucin and glycogen. Using immune electron microscopy, we have shown for the first time that only the serous cells of these developing glands contain lingual lipase. We have confirmed the findings of other investigators that these mixed glands appear prior to the development of the serous glands (1). A source of lipase is extremely important to the neonatal rat, as the process of suckling begins immediately after birth. We have noted that the mixed glands become progressively mucous in nature until they obtain their adult appearance by postpartum day 14. Lingual lipase secreted by mixed glands begins the process of neonatal fat digestion until the serous glands fully develop and assume a dominant role in secretion of the enzyme. The mixed glands differentiate into almost exclusively mucous glands, in which the demilune cells alone contain lingual lipase.

ACKNOWLEDGMENT

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Isoprenoid Fatty Acids From Marine Sponges. Are Sponges Selective?

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The burrowing sponges *Anthosigmella varians* and *Spheciospongia vesparium* were found to be rich in the isoprenoid phospholipid fatty acid 4,8,12-trimethyltridecanoic (5.2% and 23%, respectively, of the total fatty acid composition), while the burrowing sponge *Chondrilla nucula* and the demosponge *Agelas dispar* contained the acid 3,7,11,15-tetramethylhexadecanoic (13.8% and 8.6%, respectively, of the total phospholipid fatty acid composition). No other isoprenoid fatty acid was found, and the two acids described in this work did not occur concomitantly in the same sponge.
Lipids 22, 767–769 (1987).

Isoprenoid fatty acids have been of special interest during recent years, in particular for the petroleum industry and the field of geochemistry (1). Speculation about the possible conversion of marine lipids to petroleums, initiated by the discovery of pristanic and phytanic acids in a California petroleum (2) and in recent and ancient sediments, initiated interest in the geological fate of these acids as possible biomarkers for the search of petroleum (1). Two important isoprenoid fatty acids have been isolated from both terrestrial and marine sources, namely the 4,8,12-trimethyltridecanoic and the 3,7,11,15-tetramethylhexadecanoic (phytanic) acids. The latter has been found in fish and marine mammal oils (3); in sheep, ox, and butterfat (2); in patients with Refsum's syndrome (4); and in halophilic bacteria (5). The 4,8,12-trimethyltridecanoic acid has been detected in herring, seal oil, sheep fat and geological samples (2). However, to the best of our knowledge, no definite study of isoprenoid fatty acids from marine sponges has been performed, albeit that some have been isolated from a few sponges. For example, the 3,7,11,15-tetramethylhexadecanoic acid (phytanic) was reported to be a 3% constituent of the total phospholipid fatty acids of the marine sponge *Aplysina fistularis* by Walkup et al, (6). On the other hand, the marine sponge *Petrosia ficiformis* was reported to contain 4% of 4,8,12-trimethyltridecanoic acid (7) but no phytanic acid. Therefore, it seems that some marine sponges are specific for phytanic acid while others are specific for the 4,8,12-trimethyltridecanoic acid; many do not possess any isoprenoid fatty acids at all. We therefore decided to scrutinize several sponges for isoprenoid phospholipid fatty acids, and herein we report the results of our investigation.

The marine sponges in this study are *Agelas dispar, Anthosigmella varians, Spheciospongia vesparium* and *Chondrilla nucula*. The latter three sponges are bona fide burrowing sponges; these are among the most interesting since they burrow into shells, rocks and corals (8). *A. varians* is one of the most variable demosponges in shape and size. *A. varians* and corals have similar habitat requirements and usually are encountered in a competitive interaction for substrate dominance. This competitive interaction results in the overgrowth and death of the coral (9). Another common West Indian sponge, *C. nucula*, can overgrow large tracts of the corals *Porites furcata* and entire colonies of *Siderastrea siderea* and *Diploria clivosa* (9). *A. dispar*, on the other hand, is not considered to be a burrowing sponge but is a common Caribbean sponge that has not received particular attention. Interesting was the finding by Cullen and Devlin (10) of a novel quaternary 9-methyladenine in this sponge. However, to the best of our knowledge, no study of the lipids of *A. dispar* has been carried out. Interesting for us were the phospholipid fatty acids of *A. dispar*, since phospholipid fatty acids from marine sponges have been shown to possess an unusual 5,9-unsaturation (11).

EXPERIMENTAL METHODS

A. dispar, A. varians, C. nucula and *S. vesparium* were collected near La Parguera, Puerto Rico, during April 1986. The sponges were washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with chloroform/methanol (1:1, v/v) yielded the total lipids. The neutral lipids, glycolipids and phospholipids were separated by column chromatography on ammonium hydroxide-treated silicic acid (100–200 mesh) using the procedure of Privett et al. (12). The fatty acyl components of the phospholipids were obtained as their methyl esters by reaction of the phospholipids with methanolic hydrogen chloride (13) followed by purification on column chromatography eluting with hexane/ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography-mass spectrometry (GC-MS) using a Hewlett Packard 5995 A Gas chromatograph-mass spectrometer equipped with a 30 m × 0.32 mm fused silica column coated with SE-54. For the location of double bonds, N-acylpyrrolidide derivatives were prepared by direct treatment of the methyl esters with pyrrolidine/acetic acid (10:1, v/v) in a capped vial (1 hr at 100 C) followed by ethereal extraction from the acidified solution and purification by preparative layer chromatography (PLC). Hydrogenations were carried out in 10 ml of absolute methanol and catalytic amounts of platinum oxide (PtO_2). Mass spectrometry results are available upon request. The mass spectra of the isoprenoid fatty acids are presented below.

4,8,12-Trimethyltridecanoic acid methyl ester. MS m/e (rel int) 270 (M+, 3), 213 (11), 157 (18), 128 (7), 113

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Abbreviations: GC-MS, gas chromatography, mass spectrometry; PLC, preparative layer chromatography; TLC, thin layer chromatography; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine; FAME, fatty acid methyl sters; ECL, equivalent chain length.

TABLE 1

Fatty Acid Composition of the Sponges

Fatty acid	Abundance (%)			
	A. dispar	A. varians	S. vesparium	C. nucula
Tetradecanoic (14:0)	3.8	17.6	1.9	1.8
13-Methyltetradecanoic	3.8	—	—	7.1
Pentadecanoic (15:0)	1.3	—	—	2.3
4,8,12-Trimethyltridecanoic	—	5.2	23.0	—
5,9-Hexadecadienoic (16:2)	—	—	—	12.1
Hexadecenoic (16:1)	—	1.2	—	20.2
14-Methylpentadecanoic	0.9	—	—	—
Hexadecanoic (16:0)	11.0	33.6	19.3	22.7
15-Methylhexadecanoic	7.6	—	—	2.0
14-Methylhexadecanoic	7.7	—	—	—
Heptadecanoic (17:0)	1.2	—	—	1.4
3,7,11,15-Tetramethyl-hexadecanoic	8.6	—	—	13.8
Octadecenoic (18:1)	7.1	15.6	17.7	4.1
Octadecanoic (18:0)	3.1	17.4	—	4.7
17-Methyloctadecanoic	0.5	—	—	—
Nonadecanoic (19:0)	9.1	—	—	—
Docosanoic (20:0)	10.4	—	12.3	—
19-Methyldocosanoic	0.5	—	—	—
Heneicosanoic (21:0)	0.5	—	1.8	—
Behenic (22:0)	13.0	2.9	1.2	1.5
Tricosanoic (23:0)	—	—	0.7	1.0
5,9-Tetracosadienoic (24:2)	2.2	—	—	—
Lignoceric (24:0)	—	traces	4.1	3.6
5,9-Pentacosadienoic (25:2)	2.7	—	2.1	—
Pentacosanoic (25:0)	—	traces	0.6	1.0
5,9-Hexacosadienoic (26:2)	5.0	6.6	15.3	—
Hexacosanoic (26:0)	—	traces	—	0.7

(7), 99 (8), 88 (8), 87 (100), 85 (15), 83 (15), 74 (32), 71 (18), 69 (23), 59 (11), 57 (26), 55 (34).

3,7,11,15-Tetramethylhexadecanoic acid methyl ester. MS m/e (rel int) 326 (M^+, 7), 311 (2), 241 (2), 213 (4), 171 (15), 143 (8), 111 (10), 101 (100), 97 (13), 85 (9), 83 (12), 75 (20), 74 (58), 71 (17), 69 (27), 57 (31).

RESULTS

Our results are shown in Table 1, where the fatty acid composition of the four sponges is presented. We can see that the sponges *A. varians* and *S. vesparium* are specific for the 4,8,12-trimethyltridecanoic acid, while the sponges *A. dispar* and *C. nucula* contain the 3,7,11,15-tetramethylhexadecanoic acid. To the best of our knowledge, no other isoprenoid fatty acid has been detected in a marine sponge. Moreover, the two isoprenoid fatty acids mentioned above do not occur concomitantly in the same sponge, i.e., the sponge has either the 4,8,12-trimethyltridecanoic acid or the 3,7,11,15-tetramethylhexadecanoic acid, but not both at the same time. Most sponges do not have isoprenoid fatty acids. We can also see from the table that principal fatty acids common to all sponges are hexadecanoic (11–34%), octadecanoic (3–17%) and behenic (1–13%). In most of these sponges, even phospholipid fatty acids (saturated and unsaturated) were the predominant ones. The most striking example was the case of *A.varians*, where even phospholipid fatty acids accounted for more than 90% of the total mixture. In general, branched fatty acids (anteiso and iso) were not abundant but odd fatty acids (saturated and unsaturated) were even less abundant. In *A. varians*, we could not detect any odd fatty acids. Isoprenoid fatty acids were present in the three burrowing sponges that we have analyzed in this work. The most striking result was observed in the sponge *S. vesparium*, where the isoprenoid fatty acid 4,8,12-trimethyltridecanoic accounted for more than 23% of the total mixture. No other sponge has been reported, to the best of our knowledge, that contains more isoprenoid fatty acids than *S. vesparium.*

Major acids from *A. dispar* were palmitic (16:0), phytanic (20:0), docosanoic (20:0) and behenic (22:0). The latter acids accounted for more than 43% of the total mixture. Three interesting unsaturated very long chain fatty acids were encountered in the *A. dispar* that we collected. These were the 5,9-tetracosadienoic (24:2), the 5,9-pentacosadienoic (25:2) and the 5,9-hexacosadienoic (26:2) acids, which accounted for 10% of the total mixture. The 5,9-unsaturation is typical of marine sponges (6,7), but it is very rare to find three consecutive acids with the 5,9-unsaturation together in the same sponge. The phospholipid mixture was shown by thin layer chromatography (TLC) to be phosphatidylcholine (PC), phosphatidylserin (PS) and phosphatidylethanolamine (PE). For the characterization of the fatty acids, capillary GC combined with

MS was used. The fatty acid methyl esters (FAME) of the 5,9 very long chain fatty acids had equivalent chain lengths (ECL) values typical of dienoic acids; for example, the 5,9-hexacosadienoic acid had a ECL of 25.42. ECL clearly expresses where a FAME elutes with respect to the series of straight chain saturated methyl esters in a temperature-programmed run. Furthermore, upon catalytic hydrogenation (PtO_2), the dienoic acids were converted to their respective saturated acids, thus excluding the possibility of any branching. To exactly locate the double bonds, pyrrolidide derivatives of the fatty acid methyl esters were then prepared, revealing a peak at m/e 180. The latter peak is typical when two double bonds are located at the 5-and 9-positions, resulting from allylic cleavage (6). Also, comparison with authentic samples from other sponges confirmed our results (6,7). Interesting to us was the presence of a FAME with an ECL value of 17.58, just between the heptadecanoic and octadecanoic acids in the FAME mixture of *A. dispar*. The mass spectrum of the methyl ester of this acid revealed a M^+= 326 corresponding to a 20:0 acid. To explain the capillary GC retention time with the MS results, multiple methyl branching should be proposed. In fact, the latter acid was inert upon catalytic hydrogenation (PtO_2), thus excluding the possibility of any unsaturation. Furthermore, the mass spectrum of the methyl ester of this acid revealed a base peak at m/e 101 (100%) and peaks at m/e 171 (15%) and m/e 241 (2%), immediately suggesting it to be the methyl ester of the known 3,7,11,15-tetramethylhexadecanoic (phytanic) acid. A GC-MS comparison with an authentic sample confirmed the results.

DISCUSSION

Isoprenoid fatty acids found to date in marine sponges are the 4,8,12-trimethyltridecanoic and the 3,7,11,15-tetramethylhexadecanoic. There is no report to date, to the best of our knowledge, of other isoprenoid fatty acids. These acids could be of geological significance since the phytanic acid, for example, could be transformed (or has been sometime in the past) to phytane and could be part of marine sediments. In other words, these acids can be potential biomarkers (1).

In analogy to Refsum's syndrome (4), the accumulation of these isoprenoid fatty acids could be due to a deficiency of α—oxidase activity. Because of a methyl substituent at carbon 3, for example, phytanic acid cannot be β-oxidized but it can undergo α-oxidation to pristanic acid. A deficiency of α-oxidation prevents the metabolism of phytanic acid and thus may result in its accumulation in the sponge.

The origin of these isoprenoid fatty acids is also worthy of investigation. They could arise from the phytol portion of chlorophyll (14) since, for phytanic acid, that has been shown to be the case. These isoprenoid fatty acids could also be of planktonic origin or they could arise from halophilic bacteria, which are known to contain such acids (15). Still another possibility is for the sponge to be synthesizing the acids from isoprene units, since these sponges seem to be selective for one acid or the other. A symbiotic relationship between the sponge and bacteria may also be taking place. A more interesting problem seems to be the specificity displayed by these sponges. Why are these sponges specific for the 4,8,12-trimethyltridecanoic acid while others are specific for the 3,7,11,15-tetramethylhexadecanoic acid? To answer the latter question as well as to generalize the specificity displayed by the sponges in this work, a number of additional sponges will have to be analyzed for the presence of these branched acids. Work is in progress towards these ends.

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Thomas R. Tosteson and Vance Vicente from La Parguera, Puerto Rico, did the collection and classification of the sponge. Dr. O. Rosario assisted in the mass spectrometry. Financial support was provided by the University of Puerto Rico, FIPI (Grant No. 86-100-12-88.0) and NIH-MBRS (Grant No. 5 S06 RR 08 102-14).

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Ether Lipids in Oncology–Welcoming Address

Otto Westphal
Max-Planck-Institut für Immunbiologie, Freiburg, Germany

Ladies and Gentlemen,

It is an honor for me to welcome you who have come from all over the world to the First International Symposium on Ether Lipids in Oncology. As one who witnessed the development of the subject over the last 20 to 25 years, allow me a short historical sketch.

In 1960, Armour & Co. in Chicago took a patent (1) for the production of stable fat emulsions that can be administered intravenously as a parenteral high-caloric nutrient for clinical use. For the dispersion of the fat particles, soybean lecithin was recommended. But such lecithin mixtures, in contact with air, rather soon acquired pyrogenic properties. The underlying mechanism was explained by autoxidation of (conjugated) fatty acid double bonds in certain lecithin molecules. Autoxidation and occurrence of undesired pyrogenicity could be prevented by partial catalytic hydrogenation. Because hydroxy- (or peroxy-) lecithins could be suspected to be the cause of pyrogenic activity and because we at the Max-Planck-Institut in Freiburg were very interested in structurally defined pyrogens for the initiation of artificial fever (2-4), we decided to attempt the synthesis of such hydroxy-lecithins and to test them for possible pyrogenic activity.

At that time, in 1960, a young and ambitious student of chemistry, Hansjörg Eibl, contacted me to ask for a diploma and doctor thesis. He agreed to enter the field of lecithin synthesis, starting with dioleoyl lecithin and planning the preparation of higher unsaturated lecithins (5). However, rather soon we were driven into other, even more interesting aspects of that field. Eibl continued his training and interest in organic chemistry and biochemistry of phospholipids, joined the groups of van Deenen in Utrecht and Lands in Ann Arbor and made phospholipids his "grand passion" (6).

At that time, in 1960, a group of investigators around the late Prof. Herbert Fischer at our Institute in Freiburg had found that complement-fixing immune reactions are accompanied by a significant overproduction of lysolecithin due to stimulation of phospholipase A activity (7). The authors suggested that lysolecithin might be the biologically active, common initiator of complement activation (8,9). Fischer, together with Paul Gerhard Munder, then found that very small amounts of exogenous lysolecithin strongly enhanced the phagocytic activity of peritoneal macrophages in vitro and in vivo (10). This finding prompted a general survey on the possible role of lysolecithins in immune reactions.

Somewhat later, stimulated by Munder and Fischer's finding, our chemical group proposed the synthesis of structurally defined lysolecithins and especially lysolecithin analogs that might differ physicochemically and, thus also biologically from the natural prototype (11-15).

Interestingly, we soon discovered that no total organic synthesis of lysolecithins had hitherto been described. Van Deenen and coworkers had applied combinations of organic-preparative and enzymatic methods and arrived at optically active preparations. All commercially available preparations were manufactured from natural lecithin mixtures by phospholipase action.

From about 1965-68, Arnold, Eibl and Weltzien synthesized a rather comprehensive series of racemic and optically active glycero- and other phospholipids (11-15), some in radiolabeled form, which more or less resembled natural lysolecithins. Eibl developed plenty of imagination and new ideas on how to synthesize hitherto unknown compounds. Special emphasis was placed on ether-analogs (13-15) because of their higher metabolic stability and longer half-life. In 1967, Weltzien and Arnold synthesized the 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (13), abbreviated ET_{18}-OCH_3, which turned out to become the model of the new ether lipid antitumor drugs.

Many of the highly purified lysolecithin analogs were given to Fischer and Munder for biological screening in vivo and in vitro. These studies started in 1967-68, years ago, and it was P. G. Munder who did most of the work on lysolecithin analogs as a new class of immunomodulators (16). Munder and his coworkers were also the first to observe the selective destruction of various tumor cells by lysolecithin analogs (17-20). Klibansky and de Vries (21) had observed selective sensitivity of human leukemic cells towards natural lysolecithin. With the many analogs at hand, Munder was able to define certain structural requirements for optimal antitumor activity, first summarized and submitted for publication in June 1976 (16).

Glycerolipids with a long-chain alkyl substituent instead of an ester group, in the 1-position of glycerol have been known for about half a century. They occur in nature, especially in fish and higher animals (see, for example, ref. 22). Thus, ether lysolecithins in principal are natural products.

I think it is fair to say that the many studies on the action of lysolecithin analogs on tumors that followed are based on those first and fundamental observations by Munder and his coworkers. Early in the studies, it became apparent that the C_{18}-ethers in position 1 of glycerol, such as ET_{18}-OCH_3 and ET_{18}-OH, were quite efficient antitumor agents (16-19). The 2-*O*-methyl derivative is now being widely used as a model type compound of this series, as will become obvious in the course of this symposium.

In the meantime, Eibl succeeded in simplifying and economizing the chemical synthesis as well as in the large-scale preparation of lysolecithin analogs, such as ET_{18}-OCH_3 and others (23).

Of considerable importance was the finding by Munder that some of the active ether compounds, such as ET_{18}-OCH_3, would be fully active when given orally (20); this greatly facilitated the start-up of clinical trials.

In our efforts to understand the mechanism of the antitumor activity of ether lipids, we all felt most stimulated by the research work of Fred Snyder's group in Oak Ridge, starting in the late '60s, on the 1-*O*-alkyl cleavage enzyme and its mode of action (24). The Snyder group showed the enzyme system to be present in many normal tissues but absent or of very low activity in neoplastic cells. This suggested a possible explanation for the accumulation of ether lipids in tumor tissues with concomitant disturbance of their membrane metabolism. Munder

and others, at that stage arrived at the proposal that two main activities of one and the same ether lipid may combine favorably to the final antitumor effect, namely ether lipid-activated macrophages would act, directly or indirectly, on ether lipid-intoxicated tumor cells (20).

Another stimulus was the announcement by Hanahan and coworkers (San Antonio) in 1979, and by others, of the purification and structural analysis of the platelet-activating factor (PAF). PAF, being a 2-*O*-acetyl-1-*O*-alkyl-ether lipid (25), is of course chemically very similar to some of the known synthetic tumoricidal ether lipids. This certainly widened general interest in the biology of ether lipids.

I would like to express my great pleasure and our satisfaction that leading and pioneering scientists in the field of ether lipids have come here together. I welcome you all and ask you to kindly allow me not to mention many names, though I would like to welcome you as members of a kind of rather young, and therefore eager and energetic, scientific family dispersed all over the world, from the US, over Europe, including Israel, to the Far East and Japan. We are also pleased, Dr. Brachwitz, that both Germanys are represented here. We are all motivated by our work in an apparently narrow field of interest. But, as we shall see, it is in reality a vast field with important, and necessary, contributions from physical chemistry, organic chemistry and biochemistry to physiology, pharmacology and biomedical research and with prospects that we, at present, can only envisage.

You know, and you will discuss, biological activities of ether lipids other than their very tumoricidal potencies. The focus on antitumor activities is certainly wise. Yet, we may be allowed to also point to other exciting biological functions of ether lipids, and one may later find such activities to be biologically interlinked or interdependent. We will have three stimulating days, and we will have ample opportunity to make new friends and renew old friendships, also outside the formal sessions.

Thanks to the organizers of this symposium, to Unger as the scientific secretary, and to the distinguished members of the Organizing Committee. Many thanks also to those institutions who have sponsored our meeting! I herewith open our Symposium.

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REVIEW

Biosynthesis and Biotransformation of Ether Lipids[1]

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Some naturally occurring as well as synthetic ether lipids are biologically active. In certain cases, the effects of these substances are enhanced, in others, they are inhibited by compounds that were isolated from natural sources or prepared by chemical synthesis. The biotransformation of natural or "unnatural" ether lipids in microorganisms, plant or animal tissue also can lead to substances that elicit biological effects. The production of such compounds through various biotechnological techniques is a field wide open for future exploration. In addition to animal cell cultures, plant cell cultures may become useful tools in biomedical studies concerned with ether lipids.
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Naturally occurring ether lipids (alkoxylipids), once considered to be "dead ends" of lipid metabolism, have been found to be intimately involved in a variety of physiological reactions (1-4). Biologically active ether lipids include compounds containing an alkyl or a 1-alkenyl moiety. The platelet activating factor, for example, a powerful lipid mediator, is a phospholipid containing an alkyl moiety (5,6), whereas, the fecapentaenes, potent mutagens, are polyunsaturated 1-*O*-(1-alkenyl)glycerols (7).

In recent years, numerous synthetic ether lipids that do not occur in nature have been prepared to find therapeutic agents such as agonists and antagonists of natural lipid mediators (8,9) and compounds that can be of value in the therapy of cancer patients (10-13).

The results of some recent studies indicate that certain biological effects are due to products that are derived, in living systems, from both natural and synthetic "unnatural" ether lipids. The biotransformations that ether lipids undergo in animal and plant cells, therefore, are of great current interest.

The present review describes briefly a few biologically active lipids containing alkyl or 1-alkenyl moieties and, in more detail, various reactions in animal and plant cells involving both natural ether lipids and those having "unnatural" structures.

NATURALLY OCCURRING ETHER LIPIDS

Neutral and ionic lipids containing an alkyl or 1-alkenyl moiety bound to glycerol at position 1 are ubiquitous though, as a rule, minor constituents of animal cells (14-16) but not of plant cells (17). All of these natural products are chiral compounds; their alkyl and 1-alkenyl moieties usually are saturated and monounsaturated and have chains predominantly of 16, 18 and 20 carbon atoms.

Neutral ether lipids include 1-*O*-alkyl-2.3-diacyl-*sn*-glycerols, 1-*O*-(1-alkenyl)-2,3-diacyl-*sn*-glycerols (neutral plasmalogens) and a variety of ether glycolipids such as 3-*O*-alkyl-2-acyl-1-*β*-D-galactopyranosyl-*sn*-glycerols (2,4,18). Ionic ether lipids are the 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines, the 1-*O*-(1-alkenyl)-2-acyl-*sn*-glycero-3-phosphocholines (choline plasmalogens), and the corresponding glycerophosphoethanolamines and glycerophosphoserines (2,4,16).

In addition to ether lipids with a single alkyl moiety, hearts of some mammalian species contain extremely small proportions of phospholipids derived from 1,2-di-*O*-alkylglycerols (19,20). And, besides alkyl and 1-alkenyl ethers of glycerol, traces of ether lipids having a diol backbone occur in nature; these diol lipids also include neutral and ionic compounds (21). Alkyl ethers (22) and 1-alkenyl ethers (23) of cholesterol are found in mammalian heart tissues. In contrast, disteryl ethers that occur in refined edible fats and oils are not natural products. These compounds are formed from sterols of plant and animal origin during the bleaching process (24,25). They are not absorbed in the gastro-intestinal tract, and their

```
              H2C—O—R                               H H
               |                            H2C—O—C=C—R
               |                             |
R'—C—O—CH                         R'—C—O—CH
   ||  |                              ||  |
   O   |                              O   |
      H2C—O—C—R''                        H2C—O—C—R''
            ||                                 ||
            O                                  O
```

1-*O*-Alkyl-2,3-diacyl-*sn*-glycerols and 1-*O*-(1-alkenyl)-2,3-diacyl-*sn*-glycerols

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        H2C—O—R                                   H2C—O—R
          |                                          |
CH3—C—O—CH                                       HO—CH
    ||    |     O                                    |     O
    O     |     ||                                   |     ||
        H2C—O—P—O—CH2—CH2—N(CH3)3                H2C—O—P—O—CH2—CH2—N(CH3)3
                |          +                             |          +
                O-                                       O-
```

1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholines and 1-*O*-alkyl-*sn*-glycero-3-phosphocholines (PAF and Lyso-PAF).

consumption does not lead to ill effects (26). It remains to be mentioned that very small amounts of 1-*S*-alkylglycerols, i.e. thio analogues of the common 1-*O*-alkylglycerols, occur in some mammalian hearts (27).

In the past, numerous reports described biological effects of ether lipids such as bacteriostatic actions, hemopoietic effects and inhibition of tumor growth (28). As a rule, these effects were obtained solely with substances isolated from animal tissues. Synthetic compounds, however, were inactive. It thus appeared reasonable to presume that the actions observed were due to impurities in the naturally occurring ether lipids.

In the last few years, several biologically active ether lipids were isolated from animal tissues. Their structures were determined, and the effects obtained with these natural compounds could be verified using structurally identical preparations that were obtained by chemical synthesis.

The results of recent studies show that naturally occurring 1-*O*-alkyl-*sn*-glycerols differing in chain length or degree of unsaturation as well as "unnatural" synthetic alkylglycerols and other ether lipids possess different biological properties. A racemic mixture of dodecylglycerol, for example, shows strong bacteriostatic effects (29). In addition, *rac*-1-*O*-dodecylglycerol induces an efficient stimulation of mouse peritoneal macrophages for ingestion. This process may be of advantage for immunological therapy (30). A homologous compound, 1-*O*-tridecyl-*sn*-glycerol, which occurs in a marine sponge, displays toxicity to goldfish, whereas its enantiomer shows no toxicity at similar concentrations (31).

The best-known naturally occurring ether lipids are the 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines—platelet activating factor (PAF, PAFacether, AGEPC). This potent lipid mediator is a mixture of compounds having predominantly saturated alkyl moieties with 16 and 18 carbon atoms (32,33). The biological effects of PAF are summarized in several recent articles (34–37) and two books (5,38). It is striking that 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines having alkyl chains of less than 16 or more than 18 carbon atoms and those having acyl chains with more than three carbon atoms are much less active than PAF itself. And, it is remarkable that both configurational and positional isomers of PAF do not exhibit the strong biological activities of the naturally occurring substance (39). A renal lipid showing an antihypertensive effect, a substance whose structure had remained unknown for over 25 years, proved to be identical with PAF (40). Lyso-PAF, i.e. 1-*O*-alkyl-*sn*-glycero-3-phosphocholines, exhibits cancerostatic properties (11,41).

Rather unusual compounds are moenomycin A, an antibiotic containing a long-chain 2-*O*-alkyl-*sn*-glycero-3-phosphoric acid component (42), and the 1(3)-2-diacylglycero-3(1)-*O*-4′-(*N,N,N*-trimethyl)homoserines (43), ether lipids occurring in some lower eucaryotes and cryptogamic plants (44). In this context, an ether lipid having a methacrylic acid moiety bound to position 1 of glycerol (45) and a series of polyether macrolides, the halichondrins, are to be mentioned (46). The former compound is present in some brown algae occurring in Japanese waters and the halichondrins are constituents of sponges found in the same habitat. One of the latter substances, halichondrin B, exhibits potent antitumor activity.

In the early 1980s, two groups of workers described the isolation of strongly mutagenic lipids from human feces (47–50). They characterized these substances as polyunsaturated 1-*O*-(1-alkenyl) glycerols (47,48); the general structure of the fecapentaenes is shown below.

```
H2C—O\/=\/=\/=\/=\/=\/R
  |
HO—CH
  |
H2C—OH
```

Fecapentaenes I and II
I: R = $-CH_3$
II: R = $-CH_2-CH_2-CH_3$

These ether lipids are potentially important factors in the etiology of cancer of the colon (7). Very recently, an acetylenic 1-alkenylglycerol, raspailyne A, was isolated from a sponge found off the coast of Brittany (51). It is believed that this unusual ether lipid exhibits herbicidal and antifungal activities.

"Darmstoff," a mixture of long-chain cyclic acetals of glycero-3-phosphoric acid, is a potent stimulator of gastrointestinal smooth muscle which may be a hypotensive agent (10). Long-chain cyclic acetals of glycerol (52) and the corresponding phosphorylated compounds (53) have been synthesized.

"UNNATURAL" ETHER LIPIDS

Compounds that have not been found yet in nature are ether lipids having alkyl or 1-alkenyl chains of less than 12 carbon atoms and those with alkyl or 1-alkenyl moieties resembling the common polyunsaturated acyl moieties with at least 18 carbon atoms. Both saturated normal-chain and branched-chain 1-*O*-alkylglycerols have been prepared by chemical synthesis (54,55). 3-*O*-alkyl-*sn*-glycerols, the enantiomers of the naturally occurring compounds, and racemic mixtures of various ether lipids have been synthesized (56,57). Unsaturated, including polyunsaturated alkylglycerols (57) and 1,2-di-*O*-alkylglycerols (58,59) have been prepared as well.

Synthetic compounds include the symmetrical 2-*O*-alkylglycerols, 1.3-di-*O*-alkylglycerols and more complex ether lipids derived therefrom. Syntheses of neutral and ionic ether lipids with a 2-*O*-alkylglycerol (Cegla, G., and Mangold, H. K., unpublished data) or a 1,3-di-*O*-alkylglycerol backbone have been described (60). These substances have found wide use in biochemical and biomedical studies (28,61). Procedures for the synthesis of 1,2,3-tri-*O*-alkylglycerols have been worked out (58,62). The latter compounds have not been detected in nature. They are isosteric to the triacylglycerols, the common "fats," but are neither hydrolyzed enzymatically nor absorbed by the gastrointestinal tract intact. For these reasons, radioactively labeled trialkylglycerols serve as model substances in studies of intestinal fat absorption (61).

Numerous glycerophospholipids (63) and glyceroglycolipids (64) containing alkyl or 1-alkenyl moieties have been prepared.

In the past few years, intensive efforts have been devoted to the development of ether lipids that function as receptor antagonists of PAF; typical examples are shown below.

Much research work has been done also on the synthesis of ether lipids that exhibit antitumor activity, such as the 1-*O*-alkylglycerophosphocholines (11), 1-*O*-alkyl-2-*O*-methylglycerophosphocholines (e.g. ET-18-OCH_3) (65,66) and a cyclic analog of the latter (SRI 68-834) (67). A series of conjugates of lipids and 1-β-D-arabinofuranosylcytosine has been described that exhibit antineoplastic activity (68,69). In addition, a thioether lipid (BM 41.440) has been shown to be a highly active cancerostatic agent (70,71).

U-66985 U-66982

U-68043 CV-3988

ONO-6240 RO 19-3704 RU-45, 703

Antagonists of PAF.

BIOSYNTHESIS AND BIOTRANSFORMATION OF ETHER LIPIDS IN THE ANIMAL CELL

The steps involved in the biosynthesis of ether lipids are well known: the long alkyl and 1-alkenyl chains are derived from long-chain fatty acids by reduction of their CoA derivatives to alcohols, probably via aldehydes. The ether bond is formed by the reaction of these alcohols with acyldihydroxyacetonephosphates. Reduction of the resulting alkyldihydroxyacetonephosphates leads to alkylglycerophosphates, which are acylated to alkylacylglycerophosphates. From these, various neutral and ionic ether lipids are derived by following the Kennedy pathway. 1-Alkenyl moieties are formed by dehydrogenation of the alkyl moieties of alkylacylglycerophosphoethanolamines, but not the dehydrogenation of alkylacylglycerophosphocholines (72–75).

This pathway is based on metabolic studies with radioactively labeled palmitic and stearic acids and the corresponding aldehydes, alcohols and alkylglycerols. It shows that the alkyl- and 1-alkenyl moieties of ether lipids are derived from long-chain fatty acids as follows:

Fatty acid → Aldehyde → Alcohol → Alkyl ether → 1-Alkenyl ether

It is striking that the alkyl and the 1-alkenyl moieties found in the ether lipids of animal cells are exclusively saturated and monounsaturated although the acyl moieties of these lipids contain large proportions of polyunsaturated chains. Feeding of polyunsaturated fatty acids or aldehydes to rats does not lead to the formation of ether lipids containing polyunsaturated alkyl and 1-alkenyl chains. After feeding rats polyunsaturated alcohols, however, polyunsaturated alkyl and 1-alkenyl moieties can be found in the ionic ether lipids of the animals' intestinal mucosa. Similarly, feeding animals polyunsaturated 1-*O*-alkylglycerols leads to ionic ether lipids having polyunsaturated alkyl and 1-alkenyl chains. The lack of polyunsaturated alkyl and 1-alkenyl moieties in lipids of normal animal tissues is obviously due to the fact that polyunsaturated fatty acids are not reduced to aldehydes and that the latter are not reduced to alcohols (76).

In the gastro-intestinal tract, large proportions of dietary 1-*O*-alkylglycerols are cleaved at the ether bond, whereby the alkyl moieties give rise to the corresponding fatty acids (77–80). The remainder is incorporated into 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols as well as 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamines (79–81). However, 3-*O*-alkyl-*sn*-glycerols are not extensively acylated (81). The formation of neutral and ionic ether lipids proceeds through a stereospecific phosphate transfer from ATP to position 3 of 1-*O*-alkyl-*sn*-glycerol (82); the resulting 1-*O*-alkyl-*sn*-glycero-3-phosphate undergoes the reactions of the Kennedy pathway. 2-*O*-Alkylglycerols are also cleaved, though to a much smaller extent (84,85). The acylation of 2-*O*-alkylglycerols to 2-*O*-alkyl-1,3-diacyl-*sn*-glycerols proceeds at a slower rate than that of 1-*O*-alkylglycerols (82–84). 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines and 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphoethanolamines are formed in small proportions (85). Ether lysophospholipids such as 1-*O*-alkyl-*sn*-glycero-3-phosphocholines and 1-*O*-alkyl-*sn*-glycero-3-phosphoethanolamines are rapidly acylated in the intestinal mucosa (86). Dietary alkylacylglycerophospholipids are hydrolyzed at the acyl ester bonds and the resulting lyso compounds are reacylated in the mucosa (86). As mentioned above, considerable proportions of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamines are desaturated to 1-*O*-(1-alkenyl)-2-acyl-*sn*-glycero-3-phosphoethanolamines, whereas 1-*O*-

SCHEME 1. Biosynthesis of ether lipids in the animal cell.

alkyl-2-acyl-*sn*-glycero-3-phosphocholines are not converted to the corresponding plasmalogens (86,87).

Dietary 1,2-di-*O*-alkylglycerols are acylated more efficiently in the digestive tract than their symmetrical isomers (88). 1,2-Di-*O*-alkylglycerophospholipids are poorly absorbed (88); dialkylglycerophosphocholines inhibit the absorption of sterols by the mucosa (89), whereas the absorption of fatty acids and bile acids is not affected (90).

Ether lipids are only minor constituents of human food and animal feed (28,91). They are certainly not the main source of the neutral and ionic ether lipids found in the intestinal mucosa and other mammalian tissues (92). Obviously, the major portion of ether lipids is synthesized in the organism as described in several recent review articles (72–75).

The precursors of the fecapentaenes are still unknown; they are likely to be rather complex compounds (50).

The metabolic interrelationship between various ether lipids and ester lipids is of great interest. Long-chain alcohols are located at the branch point in the biosynthesis of alkyl and 1-alkenyl moieties of ether lipids as well as the alkyl and acyl moieties of waxes and other ester lipids. Therefore, the competing pathways leading to the formation of alkyl, 1-alkenyl and acyl moieties in the lipids of mammalian tissues can be assessed by following the distribution of intravenously administered long-chain alcohols from the blood to various organs of an animal. The results of such a study in the rat revealed that oxidation of the long-chain alcohol and incorporation of the resulting fatty acid in a wide variety of lipids are by far the most prominent reactions (93). Acylation of the long-chain alcohol occurs especially in the liver, which appears to be the major site of biosynthesis of wax esters. Incorporation of the long-chain alcohols into ether lipids occurs in all tissues studied, but most prominently in the heart. The biosynthesis of ether lipids continues to increase for 24 hr in all tissues except the intestine. This finding attests to the remarkable metabolic stability of ether lipids.

The results of experiments aimed at determining the relative metabolic stability of various diradylglycerophospholipids in Ehrlich ascites cells of tumor-bearing mice show that the alkylacylglycerophospholipids have a faster turnover rate than the 1-alkenylacylglycerophospholipids (94,95). Moreover, highly unsaturated molecular species of alkylacylglycerophospholipids and 1-alkenylacylglycerophospholipids exhibit higher turnover rates than those having only one or two double bonds. It is evident that ether glycerophospholipids are intimately involved in the metabolism of arachidonic acid and other highly unsaturated fatty acids that serve as precursors of various icosanoids (96).

Minor proportions of 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols and 1-*O*-(1-alkenyl)-2,3-diacyl-*sn*-glycerols are found in perinephric fat, bone marrow, the aorta and other healthy human tissues (97,98). In perinephric fat and subcutaneous adipose tissue, these two classes of neutral ether lipids occur at levels of about 0.3%, each (98,99). The content of neutral and ionic ether lipids in the human aorta is less (100); it increases, though, with age and with severity of atherosclerosis (101,102). Both transplantable and spontaneous animal tumors contain much larger proportions of neutral ether lipids and also elevated levels of ionic ether lipids, particularly plasmalogens (102,103). As a rule, the level of alkyldiacylglycerols in human tumors is about 10 times higher than in healthy tissues; it ranges from 1 to 5% (104,105). The proportions of ether lipids increase with tumor growth (106,107). Tissue remote from a tumor contains normal levels of alkyldiacylglycerols, whereas areas surrounding the tumor may exhibit fairly high levels due to protrusions of neoplastic cells (108).

Healthy tissues that have a low level of ether lipids, such as the liver, contain an enzyme that catalyzes the oxidative cleavage of the ether bond in alkylglycerols (109) as well as in ether lipids having a hydroxy group in position 2 and a hydroxy or a phosphorylcholine or phosphorylethanolamine moiety in position 3 of the glycerol backbone (110). In comparison to healthy tissues, tumors show low levels of such etherase activity (111). This difference in ether cleaving activity and differences in the levels of phospholipases C and D in healthy and neoplastic cells may be the basis for a successful cancer therapy by means of certain ether lipids (112,113).

Much work has been devoted to the characterization of tumor lipids (oncolipids) (105–107). Attempts have been made to develop diagnostic procedures based on the ether lipid contents of blood and other tissues. The validity of results obtained by photodensitometry of charred fractions on thin-layer chromatograms has been questioned (114). Better methods for the quantitative analysis of ether lipids in total lipid extracts have been developed in recent years (114–117). Nevertheless, it is not certain whether determination of ether lipid levels in tissues will be of use in the diagnosis of cancer.

It should be possible to give labeled precursors of ether lipids to cancer patients in order to permit the scintigraphic detection of neoplastic tissue because of its elevated ether lipid content. A long-chain alcohol such as (*Z*)-9-octadecenol is not suitable for this purpose as large proportions of the precursor are oxidized to the corresponding fatty acid, which is incorporated into ether lipids and ester lipids (118). Alcohols with less than 16 carbon atoms may be better suited as tumor cells incorporate these medium-chain precursors preferentially into both alkyl and 1-alkenyl moieties of ether lipids if a mixture of alcohols differing in chain lengths is administered to ascites cells (119).

It appears that the interrelation of the metabolic reactions involved in the regulation of the levels of ether lipids in the cell still has to be clarified. There is hope that tests for tumor-specific compounds in blood will permit an early diagnosis of occult primary neoplasias. The search for such tumor markers is gaining momentum (120). In this connection, a screening method based on water-suppressed proton nuclear magnetic resonance spectroscopy of plasma is of great interest (121).

Animal cell cultures are useful tools in studying the biosynthesis and biotransformation of ether lipids (61, 122–124). In general, the pathways found in animals for the biosynthesis and the metabolism of ether lipids are also operational in cultured cells that have been derived from normal and neoplastic tissues (125–127). Consequently, neutral and ionic ether lipids, predominantly alkyldiacylglycerols, alkylacylglycerophosphocholines and 1-alkenylacylglycerophosphoethanolamines, are found in normal and neoplastic cells in culture (127,128). High proportions of neutral ether lipids, which are known to be characteristic constituents of cancer cells (61,72),

occur also in normal tracheal cells when grown in culture (128).

Elevated levels of ether lipids, particularly alkyldiacylglycerols, are correlated in transformed cell lines with high rates of glycolysis. This also is obvious from the relationship between the enhanced rate of glucose uptake and metabolism and the high levels of alkyldiacylglycerols that were observed in rat squamous carcinoma cells in culture (126,129).

Oncogenically transformed fibroblast cells of mice were found to contain elevated levels of neutral lipids including alkyldiacylglycerols (130,131) and to incorporate high proportions of radioactivity into these ether lipids when incubated with labeled hexadecanol (132). Moreover, it is evident that a close relationship exists between the lipid composition of these fibroblast cells and their tumorigenicity. Thus, the cells containing the highest level of alkyldiacylglycerols produce more tumors, and the number of cells that are required for the induction of neoplasia is far less than of cell lines with low levels of ether lipids (130,133).

In addition, fibroblast cells containing high proportions of ether lipids show resistance to cell membrane fusion induced by polyethyleneglycol (130,131,133). In this context, it is interesting to note that the cellular resistance to vinblastine found in cultured leukemic lymphocytes is closely correlated with increasing proportions of alkylacylglycerophosphocholines and 1-alkenylacylglycerophosphoethanolamines as well as a concomitant decrease in diacylglycerophosphocholines and diacylglycerophosphoethanolamines (134).

Incubation of some normal and neoplastic cells in culture with 1-*O*-alkylglycerols leads to both neutral and ionic ether lipids (Fig. 1), whereas 2-*O*-alkylglycerols are incorporated preferentially into neutral ether lipids (85, 135,136). Enrichment of ionic ether lipids in cell membranes by supplementation of cell cultures with alkylglycerols may be helpful for investigating their biochemical and biophysical effects and their influence on cellular resistance (134). Furthermore, neoplastic cells in culture are useful models for studying antitumor properties of synthetic alkyllysoglycerophospholipids (12,137–139).

The Zellweger syndrome involves a defect in the biosynthesis of ether lipids (140). Methods for the biochemical diagnosis of this disease are based on the application of radioactively labeled precursors of ether lipids in human tissues (141) and fibroblast cultures (142,143).

BIOTRANSFORMATION OF ETHER LIPIDS IN THE PLANT CELL

It appears that most plant cells are devoid of ether lipids; however, a few exceptions to the rule are known (17,144). Plants are provided with an enzyme system that reduces the CoA derivatives of long-chain fatty acids to aldehydes and the latter to alcohols (145). In plants, such long-chain alcohols are acylated to wax esters (146), but ether lipids are not formed. Plants are capable, however, of incorporating exogenous alkylglycerols into more complex ether lipids (147) and converting 1,2-di-*O*-alkyl-3-*O*-β-D-galactopyranosyl-*sn*-glycerol, a synthetic ether analog of monogalactosyldiacylglycerol, to the 6-*O*-acyl derivative (148).

When long-chain fatty acids such as stearic, oleic and linoleic acids are added to heterotrophic or photomixotrophic plant cell cultures, these acids are incorporated into the cells' diacylglycerophosphocholines and other ionic ester lipids (149,150). The corresponding 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines, 1-*O*-(1-alkenyl)-2-acyl-*sn*-glycero-3-phosphocholines and other ionic ether lipids are not formed (150). It appears that in plant cell cultures neither saturated and monounsaturated fatty acids nor polyunsaturated ones are reduced to long-chain aldehydes and alcohols.

When long-chain alcohols are incubated with plant cells in culture under aerobic conditions, these alcohols are largely oxidized to the corresponding fatty acids. Under nearly anaerobic conditions, wax esters are produced in fairly large proportions (151). Ether lipids are neither formed under aerobic nor under anaerobic conditions. Obviously, plant cells in culture are neither provided with enzymes that could reduce the CoA derivatives of long-chain fatty acids (to alcohols) nor provided with those capable of catalyzing the formation of ether bonds by the reaction of alcohols with acyldihydroxyacetonephosphates. These findings and the presence of an ether-cleaving enzyme explain why plant cells in culture are devoid of ether lipids (152).

In photomixotrophic cell suspension cultures of rape, 1-*O*-alkylglycerols can serve as precursors of more complex ether lipids (152–154). When such cultures are incubated with *rac*-1-*O*-alkylglycerols, fair proportions of

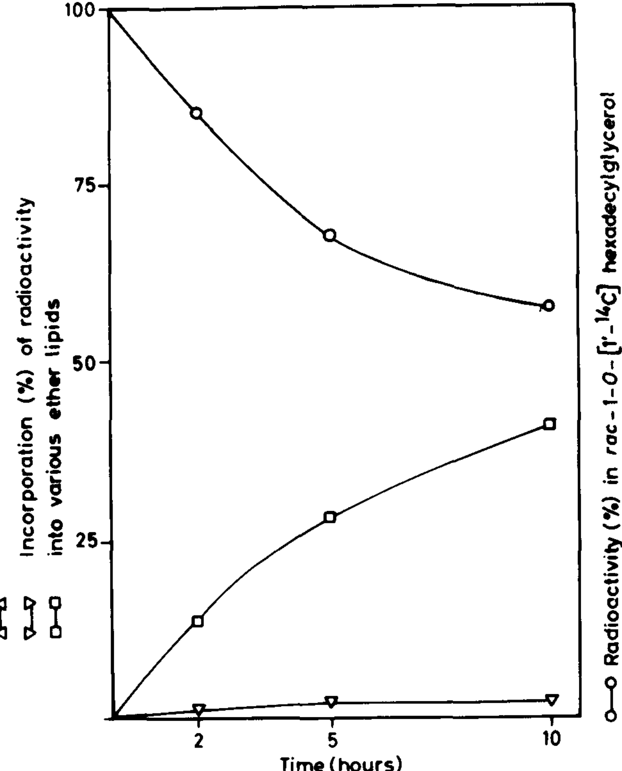

FIG. 1. Incorporation of *rac*-1-*O*-[1'-^{14}C]hexadecylglycerol into ether lipids of Ehrlich ascites cells in culture (○—○, *rac*-1-*O*-[1'-^{14}C]hexadecylglycerol, substrate; △—△, alkyldiacylglycerols; □—□, glycerophospholipids) (135).

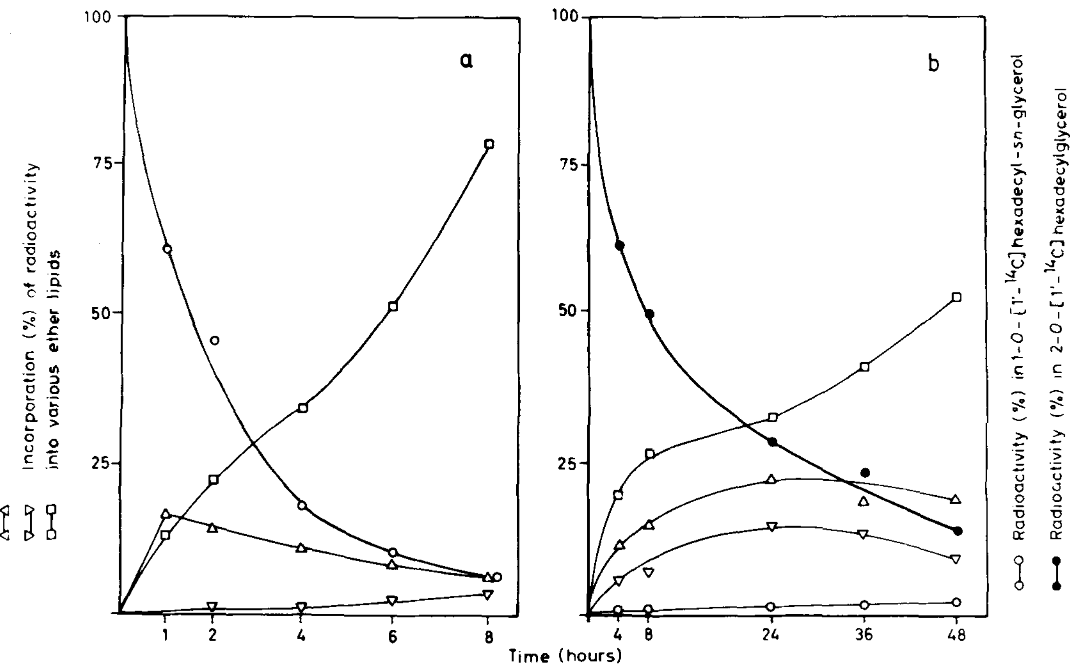

FIG. 2. Incorporation of radioactive alkylglycerols into ether lipids of photomixotrophic rape cells in suspension culture. (a) Incorporation of 1-*O*-[1'-^{14}C]hexadecyl-*sn*-glycerol (substrate) ○—○; (b) incorporation of 2-*O*-[1'-^{14}C]hexadecyl-*sn*-glycerol (substrate) ●—●; △—△, alkylacylglycerols; ▽—▽, alkyldiacylglycerols; □—□, alkylacylglycerophosphocholines (156,157).

1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines, 1-*O*-alkyl-2-acyl-*sn*-glycerols and *x*-1-*O*-alkyl-3-acylglycerols are formed. Better yields are obtained by incubating photomixotrophic rape cells with 1-*O*-alkyl-*sn*-glycerols. The synthetic enantiomers, i.e. 3-*O*-alkyl-*sn*-glycerols, are not incorporated into ionic ether lipids.

The biotransformation of various 1-*O*-alkyl-*sn*-glycerols into alkylacylglycerophosphocholines is dependent on both the chain length and the degree of unsaturation of their alkyl chains: 1-*O*-Tetradecyl-*sn*-glycerol is incorporated more efficiently than 1-*O*-hexadecyl-*sn*-glycerol and the latter more efficiently than 1-*O*-octadecyl-*sn*-glycerol. Monounsaturated and polyunsaturated 1-*O*-alkylglycerols are better substrates than saturated compounds having the same chain length.

It is certainly of interest that plant cell cultures can also acylate, phosphorylate and aminate the prechiral 2-*O*-alkylglycerols to yield chiral 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines (154).

The enzymes involved in the biosynthesis of molecular species of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines, of 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines and of 1,2-diacyl-*sn*-glycero-3-phosphocholines show similar specificities with regard to chain lengths and degree of unsaturation of the alkyl and the acyl moieties. The different alkylacylglycerophosphocholines formed in plant cell cultures are capable of replacing up to one-half of the physiological membrane lipids, i.e. diacylglycerophosphocholines, without changing the total amount of diradylglycerophosphocholines as compared to untreated cells (155). Thus, the CDP phosphocholine:diacylglycerol phosphotransferase in plants does not appear to have an absolute specificity for diacylglycerols, but also it will accept 1-*O*-alkyl-2-acyl-*sn*-glycerols and 2-*O*-alkyl-1-acyl-*sn*-glycerols as substrates.

In addition, minor proportions of neutral ether lipids such as 1-*O*-alkyl-2-acylglycerols, 1-*O*-alkyl-3-acylglycerols and 1-*O*-alkyl-2,3-diacylglycerols are synthesized by plant cells from exogenous 1-*O*-alkylglycerols (156), whereas 2-*O*-alkylglycerols are incorporated into 2-*O*-alkyl-1(3)-acylglycerols and 2-*O*-alkyl-1,3-diacylglycerols in significant quantitites (Fig. 2) (157). Similar results are obtained with cultured liver cells (85) and Ehrlich ascites cells using 1-*O*-alkylglycerols or 2-*O*-alkylglycerols as substrates (Fig. 1) (135).

In contrast to animal cells, plant cells do not dehydrogenate alkyl moieties to 1-alkenyl moieties (148,154). Yet, if added to plant cell cultures, 1-*O*-(1-alkenyl)glycerols are incorporated, though in poor yields, into ionic ether lipids (154).

It would be very helpful if a biotechnological process could be developed for the production of chiral 1-*O*-alkyl-2-*O*-methyl-*sn*-glycero-3-phosphocholines. Attempts to prepare such compounds by adding 1-*O*-alkyl-2-*O*-methylglycerols to plant cell cultures so far have failed.

The 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines formed in plant cell cultures from exogenous 1-*O*-alkylglycerols can be subjected to alkaline hydrolysis to yield the corresponding lyso compounds, 1-*O*-alkyl-*sn*-glycero-3-phosphocholines, which are of interest as cancerostatic agents. Acetylation of the latter affords 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines, PAF, in high yields (156). The biological activity of PAF obtained by this semisynthetic procedure is at least as high as that of synthetic preparations.

Similarly, 2-*O*-alkyl-*sn*-glycero-3-phosphocholines and 2-*O*-alkyl-1-acetyl-*sn*-glycero-3-phosphocholines can be

obtained from 2-*O*-alkylglycerols using the biosynthetic capacity of plant cell cultures (157). These chiral ether lipids are rather difficult to prepare by chemical synthesis.

The method described is recommended especially for the preparation of radioactively labeled chiral ether lipids. The specific radioactivities of the precursors are not diminished in the final products because there are no endogenous ether lipids that would lead to isotope dilution.

Little is known regarding the effects exogenous ether lipids can exert on plant cells. Reportedly, 1-*O*-(*Z*)-9'-octadecenyl-*sn*-glycerol, selachyl alcohol, promotes the growth of pea stem sections (158); PAF strongly stimulates ATP dependent H^+ transport in microsomes of hypocotyls (159) and the activity of microsomal 1,3-β-D-glucan synthase (160).

DISCUSSION

The rather small differences between the structures of ether lipids and ester lipids are reflected in pronounced diversities in the physical properties of liposomes formed by these substances and of membranes containing them (161). Ether lipids are more stable against chemical and enzymatic attack than ester lipids (54,55), but they are more susceptible to autoxidation (162).

The physical and chemical characteristics distinguishing the ether lipids from the structurally related ester lipids can be of use in biomedical research, clinical diagnosis and therapy. For example, cholesteryl alkyl ethers and retinyl alkyl ethers can serve as models of ester lipids in studies of lipoprotein metabolism (163). Trialkylglycerols labeled with ^3H or ^{14}C as well as a ^{75}Se-analog are used in the diagnosis of chronic pancreatitis and other disorders of fat absorption in man (61). The incapsulation of drugs into liposomes formed by ionic ether lipids and the intravenous administration of these vesicles holds great promise in cancer therapy (164).

It is striking that various biological effects of ether lipids are restricted to one or just a few molecular species of a given class of compounds such as the 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (35-38). The enormous specificity of individual compounds and the mechanisms of their biological effects remain to be clarified.

Biotransformations of ether lipids and catabolic events may play a role in the action of these substances in biological systems. It is of great interest that biotransformations of alkylglycerols and the enzymatic cleavage of the ether bond, reactions that occur in animal cells, also proceed in plant cells (Figs. 1 and 2). It thus is possible to prepare complex ether lipids including chiral compounds, from simple precursors using plant cells that do not contain endogenous ether lipids. For the same reason, it is much easier to investigate the biotransformation of natural or synthetic ether lipids in plant cell cultures than in animal cell cultures. Thus, plant cell cultures may become useful tools in biomedical studies on ether lipids.

It should be remembered that microorganisms in extreme environments contain complex ether lipids whose structures differ from those in animal cells. Halophilic bacteria, for example, lack significant proportions of ester lipids, their membrane lipids being derived from 2.3-di-*O*-phytanyl-*sn*-glycerol (165,166). Thermophilic bacteria, on the other hand, are characterized by a range of complex phospholipids and glycolipids whose structures are based on dialkyldiglycerol tetraethers and a variety of ether lipid cores (167,168). Methanogenic bacteria contain diglycosyl derivatives of dibiphytanyl tetraether and the corresponding glycerophosphoglycolipids (169). The alternative membrane lipids of certain bacteria are not necessarily the result of environmental adaptation, "but rather a consequence of evolution from a common archaebacterial ancestor" (170)—a provocative and ingenious thought, indeed!

The isolation and identification of archaebacterial lipids, their modification by chemical reactions or biotransformation, and the assessment of the biological effects of natural and synthetic compounds containing ether bonds are of great interest.

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The Degradation of Platelet-Activating Factor and Related Lipids: Susceptibility to Phospholipases C and D[1]

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1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($ET\text{-}18\text{-}OCH_3$) is an ether-linked lipid that exhibits selective cytotoxicity toward several types of tumor cells and is relatively inactive toward normal cells under the same conditions of treatment. The mechanism of this selective cytotoxicity is unknown. We conducted studies to determine whether this compound is metabolized by phospholipases C and D and, if so, whether sensitive and resistant cells differ in their ability to degrade $ET\text{-}18\text{-}OCH_3$ by these enzymes. We have examined the metabolism of the L-isomer of $ET\text{-}18\text{-}OCH_3$, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (L-$ET\text{-}18\text{-}OCH_3$), by lysophospholipase D of rat liver microsomes and by a phospholipase D from the marine bacterium *Vibrio damsela*. The metabolism of L-$ET\text{-}18\text{-}OCH_3$ was also examined in cell culture using Madin-Darby canine kidney cells, human promyelocytic leukemia cells and human myelocytic leukemia cells. In these studies, L-$ET\text{-}18\text{-}OCH_3$ and related 1-*O*-alkyl-linked phosphocholine analogs radiolabeled with ^3H in the 1-*O*-alkyl chain were used.

L-$ET\text{-}18\text{-}OCH_3$ was not hydrolyzed by lysophospholipase D from rat liver microsomes under conditions where cleavage of 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine was observed. However, phospholipase D from the marine bacterium *V. damsela* readily hydrolyzed L-$ET\text{-}18\text{-}OCH_3$ to 1-*O*-[^3H]octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate, demonstrating that L-$ET\text{-}18\text{-}OCH_3$ can be degraded by a phospholipase D. Platelet-activating factor (PAF) and lyso-PAF were also substrates for the bacterial phospholipase D.

When intact cells were incubated with radiolabeled L-$ET\text{-}18\text{-}OCH_3$, a product was formed that was identified as 1-*O*-[^3H]octadecyl-2-*O*-methyl-*sn*-glycerol. There are two mechanisms that could account for the appearance of this product. The first involves cleavage of the compound by a phospholipase C, resulting in direct release of the diglyceride. The second possible mechanism involves cleavage by a phospholipase D to form the phosphatidic acid analog with subsequent hydrolysis to the diglyceride by a phosphohydrolase. Preliminary data support the phospholipase C–type mechanism. Regardless of which mechanism operates in intact cells, the metabolic degradation of L-$ET\text{-}18\text{-}OCH_3$ does not appear to be a significant factor in the selective cytotoxicity of this antitumor agent.

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Abbreviations: GPC, glycero-3-phosphocholine; $ET\text{-}18\text{-}OCH_3$, 1-*O*-octadecyl-2-*O*-methyl-*rac*-GPC; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-GPC; lyso-PAF, 1-*O*-hexadecyl-*sn*-GPC; TLC, thin layer chromatography; MDCK, Madin-Darby canine kidney; HL60, human promyelocytic leukemia; K562, human myelocytic leukemia; PBS, phosphate-buffered saline; BSA, bovine serum albumin; ID_{50}, the concentration of compound resulting in 50% loss of viability of cells treated 48 hr.

1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (GPC) ($ET\text{-}18\text{-}OCH_3$) is an antitumor agent that is selectively cytotoxic to several types of neoplastic cells and is characterized by its relative inactivity toward normal cells (1). Of the three cell lines used in this study, HL60 cells appear to be most sensitive to $ET\text{-}18\text{-}OCH_3$, while MDCK and K562 cells are relatively resistant. It has been suggested that the selective cytotoxicity of the drug results from its accumulation in neoplastic cells containing a low activity of the alkyl cleavage enzyme (2–5). According to this hypothesis, normal cells and some types of neoplastic cells were suggested to be resistant to ether lipids due to a higher activity of the alkyl cleavage enzyme, which might prevent accumulation of the alkyl lysophospholipid analogs, thus averting cell death. However, recent studies indicate that the specific activity of the alkyl cleavage enzyme in both sensitive and resistant cells is of the same order of magnitude, suggesting that this enzyme is not responsible for the selectivity of $ET\text{-}18\text{-}OCH_3$ (6).

In the present study, we have examined alternative mechanisms of $ET\text{-}18\text{-}OCH_3$ hydrolysis that involve phospholipases C and D. Cleavage by a phospholipase C should result in the formation of a diglyceride and a phosphobase. Cleavage by a phospholipase D produces phosphatidic acid and a base. The existence of mammalian phospholipase C that degrades phosphatidylcholine has been demonstrated in Madin-Darby canine kidney (MDCK) cells (7), 3T3-L1 cells (preadipocytic), human promyelocytic leukemia (HL60) cells (8), canine myocardium (9) and HeLa cells (10). A mammalian lysophospholipase D enzyme specific for phosphocholine- and phosphoethanolamine-linked glycerolipids that contain an alkyl linkage at the *sn*-1 position and a free hydroxyl moiety at the *sn*-2 position has also been documented (11–13). We have here examined the possible involvement of phospholipases in the mechanism of action of $ET\text{-}18\text{-}OCH_3$. Lysophospholipase D, phospholipase D and phospholipase C were examined for their possible role in $ET\text{-}18\text{-}OCH_3$ hydrolysis.

Previous studies on the biological effects of $ET\text{-}18\text{-}OCH_3$ have used mixtures of the D- and L-isomers; however, we have prepared the stereochemical homolog of naturally occurring phospholipids, 1-*O*-[^3H]octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (L-$ET\text{-}18\text{-}OCH_3$), for use in studies on $ET\text{-}18\text{-}OCH_3$ metabolism.

MATERIALS AND METHODS

Cells. MDCK cells and cell culture reagents were purchased from Flow Laboratories (Rockville, MD). HL60 and human myelocytic leukemia (K562) cells were obtained from American Type Culture Collection (Rockville, MD). Liver microsomes were prepared using a Sprague-Dawley rat.

Reagents. Bovine serum albumin (BSA) and Tris-HCl were obtained from Sigma Chemical Co. (St. Louis, MO). $MgCl_2$ was purchased from Fisher Scientific Co. (Fair

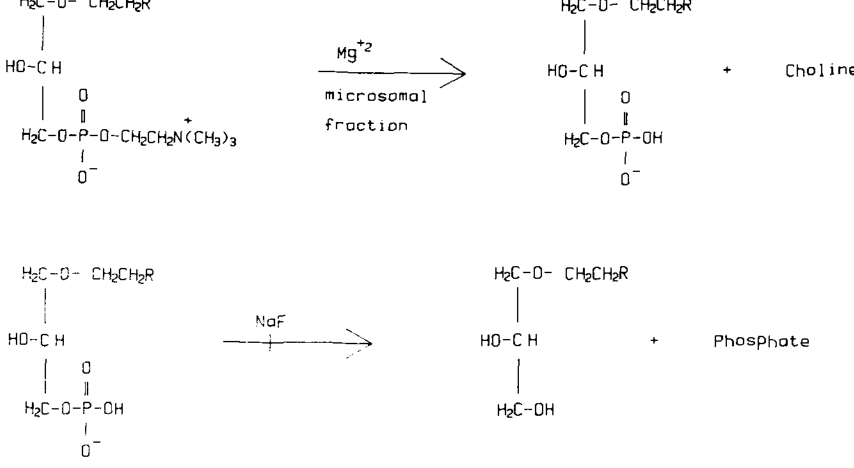


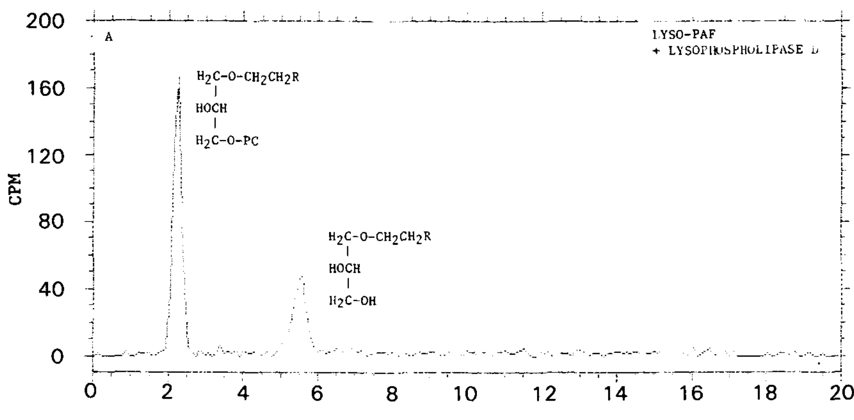
FIG. 1. Lysophospholipase D pathway. In this pathway, the initial product is 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate, which is rapidly converted by a phosphohydrolase in the microsomes to 1-*O*-alkyl-glycerol. The phosphohydrolase can be inhibited by NaF, resulting in the accumulation of 1-*O*-alkyl-*sn*-glycero-3-phosphate. R = $-(CH_2)_{13}CH_3$ or a similar long chain moiety.

Lawn, NJ). Dulbecco's phosphate-buffered saline (PBS) was from Gibco Laboratories (Chagrin Falls, OH), and p-bromophenacyl bromide was from Aldrich Chemical Co. (Milwaukee, WI).

Lipids. 1-*O*-Octadecyl-2-*O*-methyl-*rac*-GPC (ET-18-OCH_3) was a gift from Dr. Wolfgang Berdel, Technical University of Munich (Munich, FRG). 1-*O*-Hexadecyl-*sn*-GPC was purchased from R. Berchtold, Biochemical Laboratory (Bern, Switzerland). 1-*O*-[9,10-^{3}H]Hexadecyl-*sn*-GPC (lyso-PAF) (56 Ci/mmol) and 1-*O*-[9,10-^{3}H]hexadecyl-2-acetyl-*sn*-GPC (PAF) (56 Ci/mmol) were synthesized as described earlier (14). 1-*O*-Hexadecyl-2-*O*-methyl-*sn*-glycerol was a gift from Jeff Surles, University of North Carolina-Chapel Hill, (Chapel Hill, NC). 1-*O*-Octadecenyl-2-*O*-methyl-*sn*-GPC was synthesized by adding a phosphocholine moiety to 1-*O*-octadecenyl-2-*O*-methyl-*sn*-glycerol according to the method of Brockerhoff and Ayengar (16). The synthesis of 1-*O*-octadecenyl-2-*O*-methyl-*sn*-glycerol paralleled the synthesis of 1-*O*-alkyl-2-*O*-ethyl-*sn*-glycerol reported previously (15), substituting selachyl alcohol (1-*O*-octadecenyl-*sn*-glycerol; Western Chemical Industries Ltd., Vancouver, Canada) for chimyl alcohol and methyl methanesulfonate for ethyl methanesulfonate. The 1-*O*-octadecenyl-2-*O*-methyl-*sn*-GPC was tritiated catalytically using palladium (10%) on charcoal to yield 1-*O*-[9,10-^{3}H]octadecyl-2-*O*-methyl-*sn*-GPC (56 Ci/mol).

Lysophospholipase D assay. The lysophospholipase D assays were performed using rat liver microsomes as described by Wykle and Schremmer (11). Protein determinations were made using the method of Bradford (Biorad, Richmond, CA) with BSA as a standard (17). The substrates tested were 1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*sn*-GPC and 1-*O*-[^{3}H]hexadecyl-*sn*-GPC. p-Bromophenacyl bromide, which did not block lysophospholipase D activity, was added to prevent *sn*-2 acylation of the lyso compound (13). The reactions were terminated by lipid extraction using an acidified Bligh and Dyer (18) procedure in which the methanol contained 2% acetic acid. After extraction, the lipids were analyzed by thin layer chromatography (TLC). Following chromatography, the resolved

FIG. 2. Substrate specificity of lysophospholipase D. Of the compounds tested, only those containing ether-linked groups at the *sn*-1 position are hydrolyzed. In addition, the 2-hydroxy group must be unesterified. PC, phosphocholine; PE, phosphoethanolamine; R = $-(CH_2)_{13}CH_3$ or a similar long chain moiety.



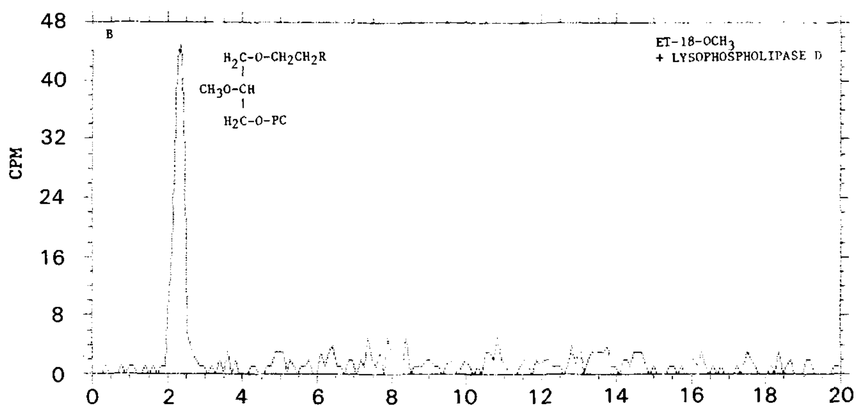


FIG. 3. Relative susceptibility of 1-*O*-[^{3}H]alkyl-2-lyso-*sn*-GPC (lyso-PAF) and 1-*O*-[^{3}H]alkyl-2-*O*-methyl-*sn*-GPC (L-ET-18-OCH_3) to rat liver lysophospholipase D. Lyso-PAF was hydrolyzed by the enzyme under the experimental conditions; L-ET-18-OCH_3 was not. Each 3-ml incubation mixture contained $MgCl_2$ (5 mM), Tris-HCl buffer (0.1 M, pH 7.1), rat liver microsomes (0.5 mg protein), p-bromophenacyl bromide (0.13 M) added directly in 20 μl acetone, and a combination of the respective unlabeled substrates (17 nmol) and radiolabeled substrates (300,000 dpm; 2 pmol) added in 20 μl ethanol. Each incubation was shaken at 37 C for 10 min, and the reactions were terminated by extraction. The lipids were then dried under N_2, resuspended in chloroform/methanol (9:1, v/v) and separated on layers of Silica Gel 60 by developing in ethyl ether/water (100:0.5, v/v) or in chloroform/methanol/glacial acetic acid/water (50:25:8:3, v/v/v/v). The samples depicted in this figure were chromatographed in the solvent system ethyl ether/water (100:0.5, v/v). The major peaks are identified by the adjacent structures, based on migration with standards. In Figures 3–5, radioactivity was measured using a Bioscan radiochromatogram imaging system and is expressed as counts per minute. The abscissa represents distance in cm on the thin layer chromatography plate. Scans of the entire sample lane are shown. Origin, 1–2 cm; solvent front, 18–20 cm.

lipids were visualized by exposing the plates to iodine vapors; radiolabeled products were located using a radiochromatogram imaging system (Bioscan Inc., Washington, DC) (Fig. 3). Regions containing radiolabeled lipid were then scraped and counted using a Packard liquid scintillation counter. Further details of the experimental procedures are given in the figure legends.

Bacterial phospholipase D assay. The phospholipase D enzyme isolated from the marine bacterium *Vibrio damsela* was a gift from A. S. Kreger (Bowman Gray School of Medicine, Winston-Salem, NC). This enzyme preparation was from the stage-4 purification pool with a specific activity of 2 × 10^6 hemolytic units/mg protein and a concentration of 1.47 mg protein/ml (19). Details of incubations are given in the legends of Figures 4 and 5. Substrates tested were 1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*sn*-GPC (1.5 × 10^6 dpm, 12 pmol), 1-*O*-[^{3}H]hexadecyl-*sn*-GPC (6.2 × 10^5 dpm, 5 pmol), 1-*O*-[^{3}H]hexadecyl-2-acyl-*sn*-GPC (9 × 10^5 dpm, 7 pmol) and 1-*O*-[^{3}H]hexadecyl-2-acetyl-*sn*-GPC (1.0 × 10^6 dpm, 8 pmol).

Incubations of ET-18-OCH_3 with intact cells. MDCK cells were cultured as a monolayer in Dulbecco's modification of Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml, 0.22% Na_2HCO_3 and 2 mM L-glutamine. HL60 and K562 cells were cultured in suspension in RPMI 1640 medium with the same supplements. For these experiments, the cells were pelleted, resuspended in medium containing radiolabeled L-ET-18-OCH_3 and cultured in 35-mm petri dishes during the 48-hr time course (Fig. 6). The concentration of L-ET-18-OCH_3 (24 nM) in these experiments was 100-fold less than the

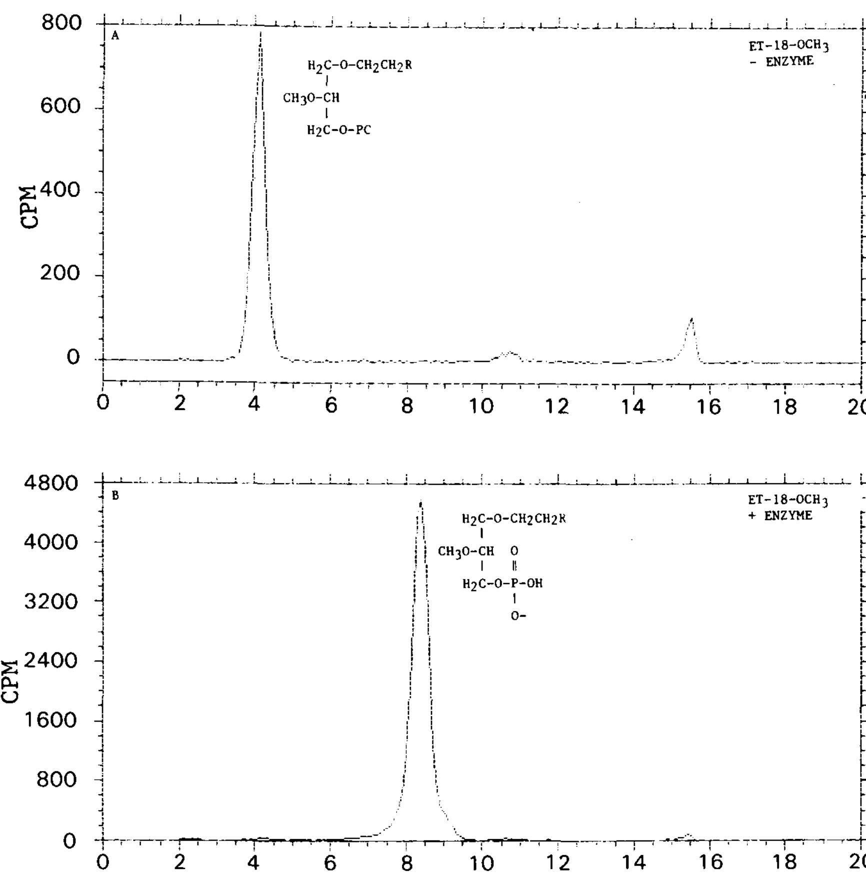


FIG. 4. Hydrolysis of 1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*sn*-GPC to 1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate by phospholipase D isolated from the bacterium *Vibrio damsela*. Each incubation mixture in a final volume of 1 ml phosphate-buffered saline was shaken for 30 min at 37 C and contained 1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*sn*-GPC (1.5 $\times$ 10^6 dpm, 12 pmol) dried under N_2 and sonicated in 400 μl PBS and (A) no enzyme or (B) 29.4 μg enzyme. The reactions were terminated by lipid extraction as described in Materials and Methods for lysophospholipase D assays. Samples were analyzed by thin layer chromatography using the solvent system chloroform/methanol/glacial acetic acid/water (50:25:8:3, v/v/v/v).

ID_{50} (the concentration of compound resulting in 50% loss of viability of cells treated 48 hr) of the sensitive HL60 cell line. After each incubation period, the cells and fluids (medium) were extracted separately using an acidified Bligh and Dyer (18) procedure in which the methanol contained 2% acetic acid. Lipids were analyzed by TLC using solvent systems described below.

RESULTS AND DISCUSSION

Hydrolysis of L-ET-18-OCH_3 by lysophospholipase D. Lysophospholipase D has been shown to hydrolyze 1-*O*-alkyl-linked choline- or ethanolamine-containing phosphoglycerides but not the corresponding 1-*O*-acyl compounds (13) (Figs. 1 and 2). The enzyme is found in a number of tissues, requires Mg^{+2} for activity and does not act on substrates esterified at the *sn*-2 position (11,12). The cellular role of this novel enzyme is unknown. In the present study, when radiolabeled 1-*O*-hexadecyl-*sn*-GPC (lyso-PAF) was incubated with rat liver microsomes in the presence of Mg^{+2}, the substrate was hydrolyzed to 1-*O*-hexadecyl-*sn*-glycerol (Fig. 3A) as reported by Wykle and Schremmer (11). It was also reported that PAF could be cleaved by lysophospholipase D if the acetate group was first removed by an acyl hydrolase present in the microsomes to form the lyso compound (13).

To establish that the enzyme under study was the Mg^{+2} requiring lysophospholipase D described by Wykle and Schremmer (11), the ion requirements of the enzyme were investigated. When the microsomal enzyme preparation was treated with 10 mM EDTA without the addition of Mg^{+2}, 1-*O*-hexadecyl-*sn*-GPC was not hydrolyzed to 1-*O*-hexadecyl-*sn*-glycerol (data not shown). This confirmed that the enzyme being assayed was the lysophospholipase D described earlier.

We tested whether L-ET-18-OCH_3 could be hydrolyzed directly by lysophospholipase D. When radiolabeled L-ET-18-OCH_3 was incubated with rat liver microsomes in the presence of Mg^{+2}, hydrolysis did not occur (Fig. 3B). These findings are in agreement with earlier observations that a free hydroxy group is required at the *sn*-2 position (11–13). Unless a mechanism is available to remove the 2-*O*-methyl group, lysophospholipase D does not appear to play a role in the metabolism of L-ET-18-OCH_3.

Hydrolysis of L-ET-18-OCH_3 by a bacterial phospholipase D. Several phosphocholine-containing lipids were

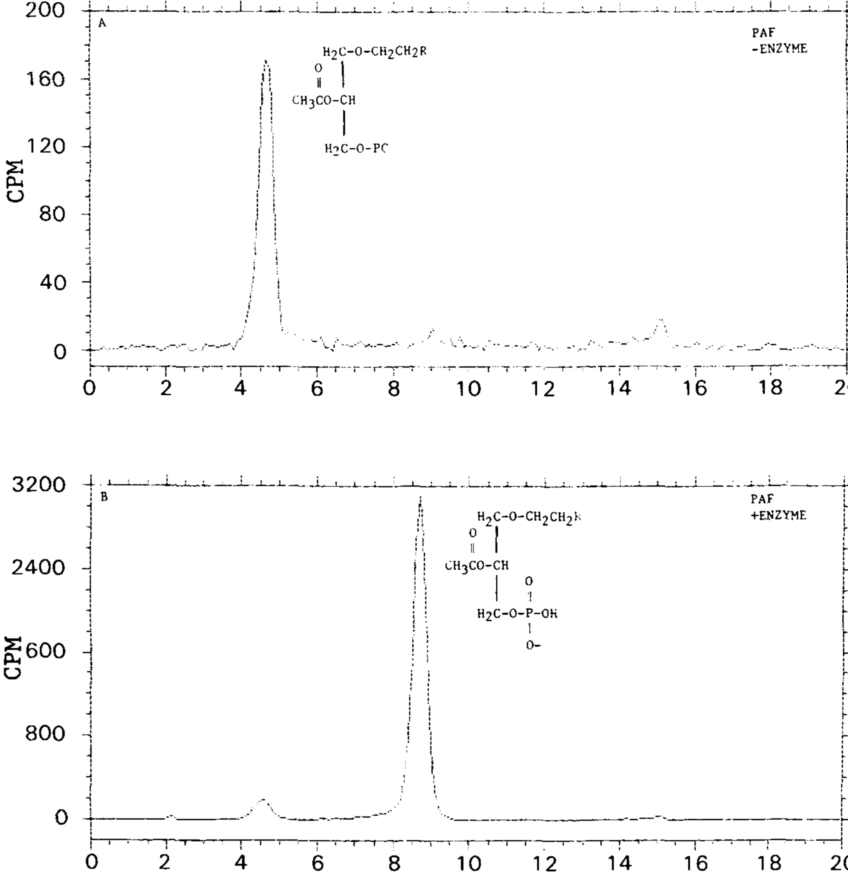


FIG. 5. Hydrolysis of 1-*O*-[3H]hexadecyl-2-*O*-acetyl-*sn*-GPC (platelet-activating factor) to 1-*O*-[3H]hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphate by bacterial phospholipase D. The incubation mixture in a final volume of 1 ml contained 1-*O*-[3H]hexadecyl-2-acetyl-*sn*-GPC (1.0×10^6 dpm, 8 pmol) added as described in Figure 4. Incubation times and other conditions as well as methods for analysis of the products were the same as described in Figure 4.

tested as substrates for phospholipase D isolated from the bacterium *Vibrio damsela* (19). Figure 4 shows the degradation of L-ET-18-OCH_3 to 1-*O*-[3H]octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate by this enzyme. This demonstrated that L-ET-18-OCH_3 is susceptible to metabolism by a phospholipase D and provided a standard for the reaction product of phospholipase D cleavage. Figure 5 shows the degradation of 1-*O*-[3H]hexadecyl-2-acetyl-*sn*-GPC (PAF) to 1-*O*-[3H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphate. Other substrates tested were 1-*O*-[3H]hexadecyl-2-acyl-*sn*-GPC and 1-*O*-[3H]hexadecyl-*sn*-GPC (lyso-PAF). These compounds were also hydrolyzed to the corresponding phosphatidic acid by the bacterial phospholipase D (data not shown).

Hydrolysis of L-ET-18-OCH_3 in intact cells. Figure 6 shows the loss of radiolabeled L-ET-18-OCH_3 from the medium and uptake by MDCK, HL60 and K562 cells. The data points represent relative amounts of label since conditions for recovery were not optimal in these studies. Loss of label due to desaturation at position 9,10 cannot be ruled out. We have not explored this possibility. We have ascribed the loss of label to adsorption to the petri dishes due to the high radiospecific activity of the [3H]L-ET-18-OCH_3 employed (56 Ci/mmol); similar low recoveries are observed in studies using ^{3}H PAF of high radiospecific activity. The concentration of [3H]L-ET-18-OCH_3 used in these studies was 24 nM, whereas the ID_{50} has been reported as 2.5 μM (20). Figure 7 shows the appearance of the hydrolysis product from each cell line. This product, which appeared in both cell and fluid extracts, was identified by TLC as the diglyceride 1-*O*-[3H]octadecyl-2-*O*-methyl-*sn*-glycerol. In two neutral lipid TLC systems, hexane/ethyl ether/formic acid (90:60:4, v/v/v) and ethyl ether/water (100:0.5, v/v), the hydrolysis product migrated (Rf = 0.5 in the ethyl ether/water [100:0.5, v/v] solvent system) with authentic 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol. A TLC zonal scan of products isolated from HL60 fluids after incubation with [3H]ET-18-OCH_3 for 48 hr showed the radiolabeled hydrolysis product in zones 16–21 (Fig. 8). The 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol standard was visualized by iodine staining or by charring the standard lanes. The Rf value of the hydrolysis product in the ethyl ether/water (100:0.5, v/v) solvent system increased after acetylation to 1-*O*-alkyl-2-*O*-methyl-3-acetylglycerol (data not shown). This demonstrated the presence of a free hydroxyl moiety in the radiolabeled hydrolysis product.

No evidence for 1-*O*-alkyl cleavage enzyme products was obtained. Alkyl cleavage activity should have resulted in the appearance of labeled fatty aldehyde and

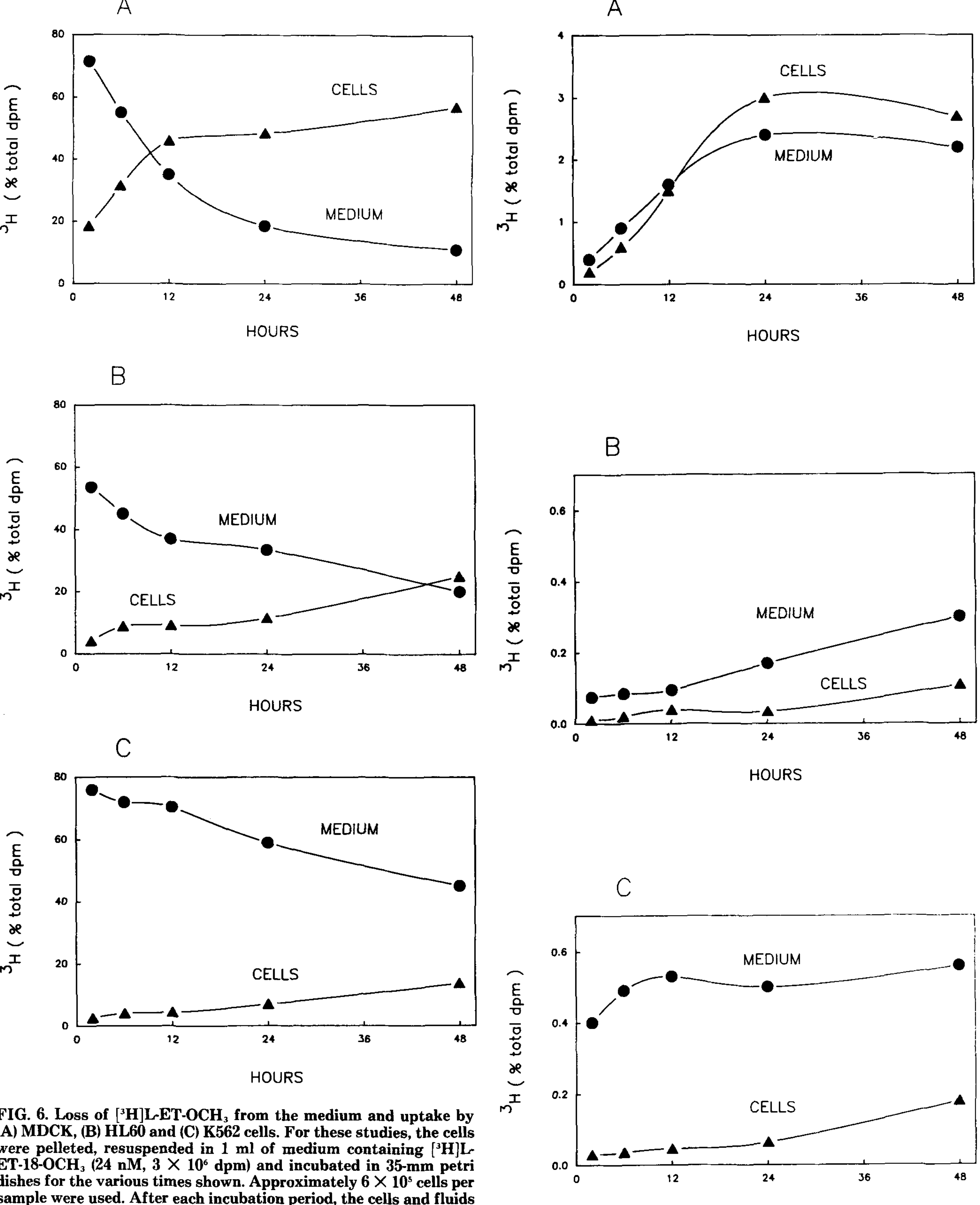


FIG. 6. Loss of [^{3}H]L-ET-OCH_3 from the medium and uptake by (A) MDCK, (B) HL60 and (C) K562 cells. For these studies, the cells were pelleted, resuspended in 1 ml of medium containing [^{3}H]L-ET-18-OCH_3 (24 nM, 3×10^6 dpm) and incubated in 35-mm petri dishes for the various times shown. Approximately 6×10^5 cells per sample were used. After each incubation period, the cells and fluids (medium) were extracted and analyzed by thin layer chromatography. The neutral lipid solvent systems used were hexane/ethyl ether/formic acid (90:60:4, v/v/v) or ethyl ether/water (100:0.5, v/v). The basic phospholipid solvent system used was chloroform/methanol/ammonium hydroxide (65:35:8, v/v/v). Values are percentage of total [^{3}H]ET-18-OCH_3 (dpm) added to each sample that was recovered in the cell extract (▲) or fluid extract (●).

FIG. 7. The formation of 1-*O*-[^{3}H]alkyl-2-*O*-methylglycerol by (A) MDCK, (B) HL60 and (C) K562 cells after incubation with [^{3}H]L-ET-18-OCH_3. These are *the same samples* as in Figure 6. Values are percentage of total dpm added to the sample that were recovered as radiolabeled diglyceride in the cell extract (▲) or fluid extract (●).

fatty acid, which would be expected to incorporate into various cellular lipids. Hoffman and Snyder (6) recently compared alkyl cleavage enzyme activity in cells that demonstrate different sensitivities to ET-18-OCH_3. In contrast to previous studies by other investigators, they found that microsomal preparations from sensitive (HL60) and resistant (K562, MDCK) cells did not differ significantly in alkyl cleavage enzyme activity. They also reported the appearance of the metabolic products 1-*O*-alkyl-2-methoxyglycerol and 1-*O*-alkylglycerol after incubation of HL60, K562 and MDCK cells for 24 hr with 0.5 μM ET-18-OCH_3.

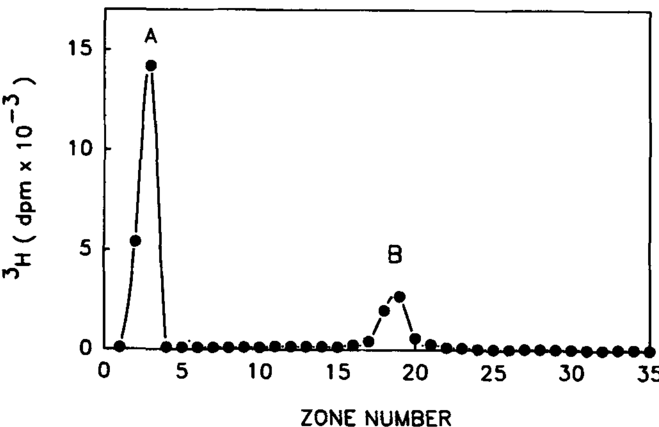


FIG. 8. Zonal scan of a thin layer chromatogram showing the migration of [^{3}H]L-ET-18-OCH_3 and the radiolabeled hydrolysis product, both isolated from HL60 fluids after incubation with [^{3}H]L-ET-18-OCH_3 for 48 hr. The labeled product at zones 16–21 migrated with authentic 1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol standard. Each zone division represents a 5-mm region of the thin layer chromatographic sample lane. The origin was at zone 3; the solvent front corresponded to zone 35. (A) [^{3}H]L-ET-18-OCH_3; (B) diglyceride (1-*O*-hexadecyl-2-*O*-methyl-*sn*-glycerol).

The appearance of a radiolabeled diglyceride metabolite suggested a phospholipase C–type mechanism of hydrolysis of ET-18-OCH_3 in whole cells. However, it was also important to consider an alternative mechanism in which a phospholipase D enzyme coupled with a phosphohydrolase could result in the appearance of a diglyceride product. The second mechanism is analogous to that of lysophospholipase D in rat liver microsomes mentioned above. Figure 9 shows the reaction scheme for each of the possible mechanisms of ET-18-OCH_3 hydrolysis.

A basic phospholipid TLC solvent system (chloroform/methanol/ammonium hydroxide [65:35:8, v/v/v]) capable of resolving phosphatidic acid from other components was used to determine if the metabolite 1-*O*-[^{3}H]octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphate was present; none was detected. This evidence supports a phospholipase C–type mechanism of hydrolysis (Fig. 9A), but further experiments must be conducted to clarify this mechanism.

These studies demonstrate that ET-18-OCH_3 is very poorly metabolized by all the cell lines tested. In addition, the liver lysophospholipase does not act on the compound. Based on earlier findings and those reported here, ET-18-OCH_3 is not a substrate for the alkyl cleavage enzyme. The pattern of uptake of ET-18-OCH_3 and release

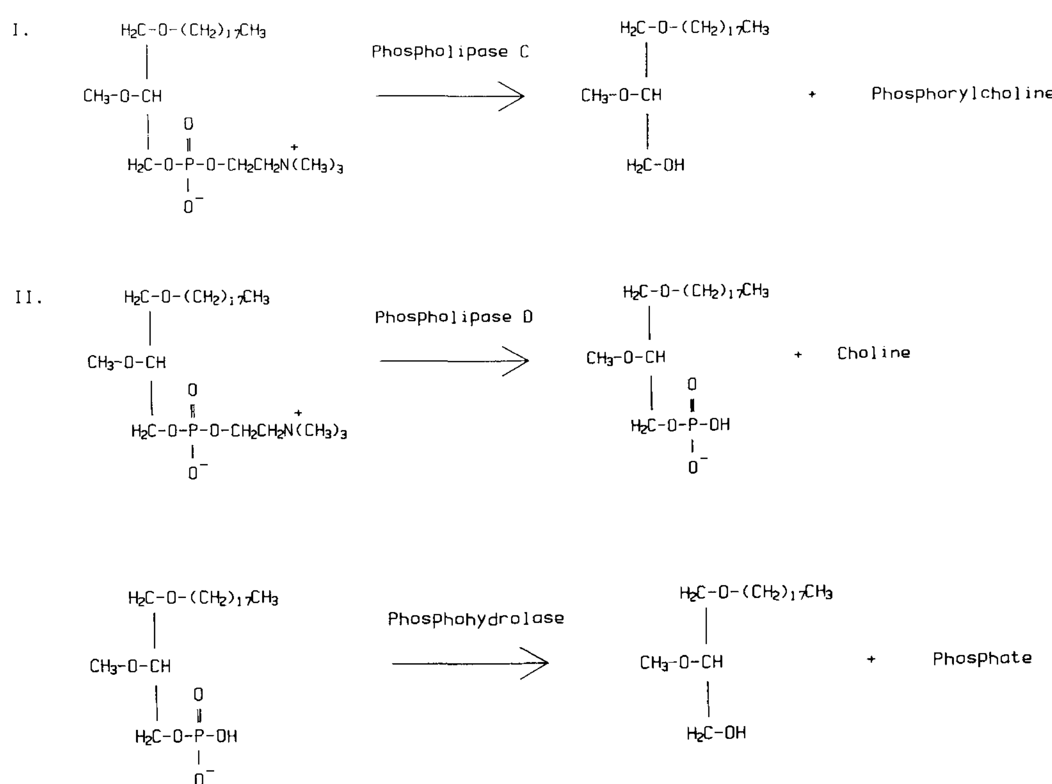


FIG. 9. Two possible pathways explaining the appearance of a radiolabeled diglyceride after incubation of cells with [^{3}H]L-ET-18-OCH_3.

of diglyceride product varied among the cell lines tested. However, these data did not suggest a correlation between cytotoxicity of ET-18-OCH_3 and metabolic degradation of this compound. The selective cytotoxicity of ET-18-OCH_3 toward tumor cells does not appear to be attributable to differences in the catabolism of the compound in normal versus neoplastic cells.

ACKNOWLEDGMENTS

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Enzymatic Acylation of Ether and Ester Lysophospholipids in Rat Liver Microsomes[1]

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The acylation of lysophospholipids by rat liver acyltransferases was studied. A comparison between ester and ether lysophospholipids as substrates revealed large differences in substrate properties. For instance, oleic acid from oleoyl-CoA and arachidonic acid from arachidonoyl-CoA were not incorporated into 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine under experimental conditions that allowed an optimal transfer of oleic acid and arachidonic acid to 1-*O*-palmitoyl-*sn*-glycero-3-phosphocholine. However, we observed an acyl-CoA-independent transfer of arachidonic acid from 1-*O*-stearoyl-2-*O*-arachidonoyl-*sn*-glycero-3-phosphoinositol to 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine.

Lipids 22, 808–812 (1987).

Acyltransferases are important enzymes for the biosynthesis of phospholipids. They catalyze the stepwise acylation of glycerophosphate (de novo synthesis) (1,2) and also are involved in the acylation of ester lysophospholipids, which are generated in natural membranes by the action of phospholipases A_1 and A_2 (3,4).

The metabolic fate of ether lysophospholipids receives increasing attention because these compounds show antineoplastic activity (5-9). Ether lysophospholipids are more toxic in cell cultures than the respective ester analogs (10). This may reflect differences in the substrate properties of ether lysophospholipids in comparison to ester lysophospholipids. However, little information exists on the substrate behavior of ether lysophospholipids for phospholipid–metabolizing enzymes.

Two possible mechanisms of acylation of lysophospholipids are considered here. First, ether lysophospholipids are reported to be acylated by acyltransferases that normally acylate ester lysophospholipids (11–14). On the other hand, specific acylation of ether lysophospholipids by CoA-dependent and CoA-independent transferases has been reported (15–18). These transferases esterify 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine by transferring an arachidonoyl residue from the 2-position of diacylphospholipids to the 2-position of the ether lysophospholipid. Both reactions, acylation and transacylation, yield identical products. In this study, we set out to determine which is the dominant pathway in the acylation of 1-*O*-octadecyl-*sn*-glycero-3-phosphocholine.

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Abbreviations: Lyso-PC, 1-*O*-palmitoyl-*sn*-glycero-3-phosphocholine; lyso-PA, 1-*O*-palmitoyl-*sn*-glycero-3-phosphate; lyso-PE, 1-*O*-palmitoyl-*sn*-glycero-3-phosphoethanolamine, lyso-PS, 1-*O*-palmitoyl-*sn*-glycero-3-phosphoserine; lyso-PI, 1-*O*-palmitoyl-*sn*-glycero-3-phosphoinositol; DLPC, 1-*O*-palmitoyl-propandiol-3-phosphocholine; CoA, coenzyme A; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid.

EXPERIMENTAL

Materials. Lysophosphatidylinositol, lysophosphatidylserine, and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were obtained from Sigma (Munich, FRG); 1-*O*-stearoyl-2-*O*-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoinositol, 1-*O*-[^{3}H]-octadecyl-*sn*-glycero-3-phosphocholine, 1-*O*-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine and [1-^{14}C]oleoyl-CoA were purchased from Amersham Buchler (Braunschweig, FRG). Lysophosphatidylcholine, lysophosphatidic acid and lysophosphatidylethanolamine were synthesized in our laboratory as described before (19,20). Oleoyl-CoA and arachidonoyl-CoA were prepared according to Reitz et al. (21). Other chemicals were of analytical or higher grade and used without further purification.

Methods. Rat liver microsomes were prepared according to Yawetz et al. (22). The microsomes were suspended in an aqueous solution of 4 M NaCl, 0.1 M Tris/HCl (pH 7.4) to yield a protein concentration of 20 mg/ml. The suspension was sonicated in a Branson microtip B 15 for 5 min (energy stage 4) using the pulse mode with 20% pulse time. After dilution with an equal volume of water, the suspension was centrifuged at 100,000 × g for 60 min in a Beckman ultracentrifuge (L5-65 B). About 80% of the acylation activity was found concentrated in a fluffy-layer fraction. Pellet and supernatant showed each about 10% acylation activity. The fluffy layer was used as an enzyme source for the acylation studies.

During acylation of lysophospholipids with acyl-CoA, free CoA is formed. The amount of CoA can be measured using DTNB as a thiol-specific reagent (23). The acylation assays were performed at 37 C in a buffer system of 100 mM Tris/HCl (pH 7.4), containing 0.2 mM DTNB and 0.15 mg/ml microsomal protein. The amount of CoA formed was monitored via DTNB at 412 nm in a Hitachi spectrophotometer 150-20. The substrate concentration was 30 nmol/ml for lyso-PA and 170 nmol/ml for the other lysophospholipids. In order to measure the hydrolysis of acyl-CoA by acyl-CoA hydrolases, 2-desoxy-lysolecithin (DLPC), which cannot be acylated, was used instead of lysophospholipid. The rate from this reaction was considered to be the blank rate and was subtracted from the rate of the acylation reaction in the presence of substrates.

In the radioactive assays, DTNB was omitted from the incubation mixture. Either 170 nmol (20 nCi) 1-*O*-[^{14}C]-palmitoyl-*sn*-glycero-3-phosphocholine or 170 nmol (1 μCi) 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine was incubated with 50 nmol (1 μCi) [1-^{14}C]oleoyl-CoA and 0.15 mg microsomal protein in 1 ml 0.1 M Tris/HCl buffer (pH 7.4). The reaction was stopped after the indicated time periods by adding 100 μl formic acid. After freeze-drying, the lipids were extracted from the reaction mixture with chloroform and were separated by thin layer chromatography (TLC) on silica gel plates (Silica Gel 60, 20 × 20 cm, Merck, Darmstadt, FRG) in a solvent system of chloroform/methanol/acetic acid/water (100:60:20:5 by vol).

TABLE 1

Acylation Rates of 1-O-Palmitoyl-*sn*-glycero-3-phosphocholine/ phosphate with Oleoyl-CoA at Different DTNB Concentrations

DTNB (nmol/ml)	Specific activity (nmol/min/mg)		
	Lyso-PC*	Lyso-PA*	DLPC
1000	111	211	12
800	111	211	13
600	119	215	14
400	125	223	16
200	134	233	18
100	134	233	20

The acylation reactions were measured at 412 nm with 1-O-palmitoyl-*sn*-glycero-3-phosphocholine/phosphate (lyso-PC/PA) as substrates. The acyl-CoA hydrolase activity was monitored with DLPC. The DTNB concentration ranged from 1 μmol to 0.2 μmol/ml in a 100 mM Tris/HCl buffer (pH 7.4) at 37 C. The protein concentration was 0.15 mg, and the reaction was started with 20 nmol oleoyl-CoA. All acylation rates (*) are corrected for hydrolase rates.

TABLE 2

Acylation of Lysophospholipids with Oleoyl- and Arachidonoyl-CoA

Lyso-PL	Specific activity (nmol/min/mg)	
	18:1	20:4
Lyso-PC	125	130
Alkyl lyso-PC	<5	<5
Lyso-PA	240	34
Lyso-PS	53	85
Lyso-PI	53	117
Lyso-PE	36	32

In an incubation volume of 1 ml 100 mM Tris/HCl (pH 7.4), 0.15 mg microsomal protein was incubated with 170 nmol alkyl lyso-PC, lyso-PC, lyso-PE, lyso-PS, lyso-PI or 30 nmol of the respective lyso-PA at 37 C. The incubation mixture contained 0.2 mM DTNB. The acylation was monitored at 412 nm by following the formation of CoA from acyl-CoA with DTNB. All data are corrected for the hydrolase reaction (18 nmol/min/mg).

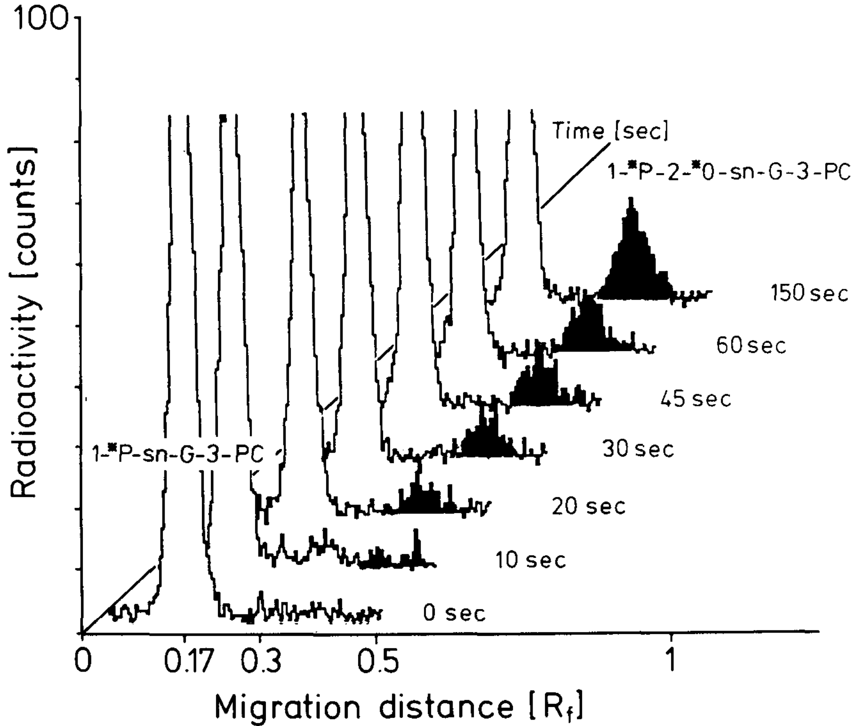

FIG. 1. Acylation of 1-O-[1-^{14}C]palmitoyl-lysolecithin with [1-^{14}C]oleoyl-coenzyme A. Microsomal protein (0.15 mg) was incubated with 170 nmol 1-O-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (1-*P-*sn*-G-3-PC) and 50 nmol [1-^{14}C]oleoyl-CoA for 0, 10, 20, 30, 45, 60 and 150 sec in an incubation volume of 1 ml. The reacylation reaction was stopped with formic acid. After freeze-drying, the lipids were extracted from the reaction mixture with chloroform and were separated by TLC on silica gel plates in a solvent system of chloroform/methanol/acetic acid/water (100:60:20:5 by vol). After drying, the plates were scanned in a Berthold linear analyzer LB 2842. The radioactive reaction product was identified as 1-O-palmitoyl-2-O-oleoyl-*sn*-glycero-3-phosphocholine (1-*P-2-*O-*sn*-G-3-PC).

After drying, the plates were scanned in a Berthold linear analyzer LB 2842 with data system LB 500. The acylation products were identified by their R_f-values and by comparison with reference substances that were run on the same plate.

For transacylation, 1-O-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (10 nmol, 7 μCi) was incubated with 8 nmol (1 μCi) 1-O-stearoyl-2-O-[1-^{14}C]arachidonoyl-phosphoinositol and 0.15 mg microsomal protein in 1 ml 100 mM Tris/HCl-buffer (pH 7.4). After 60 min, the

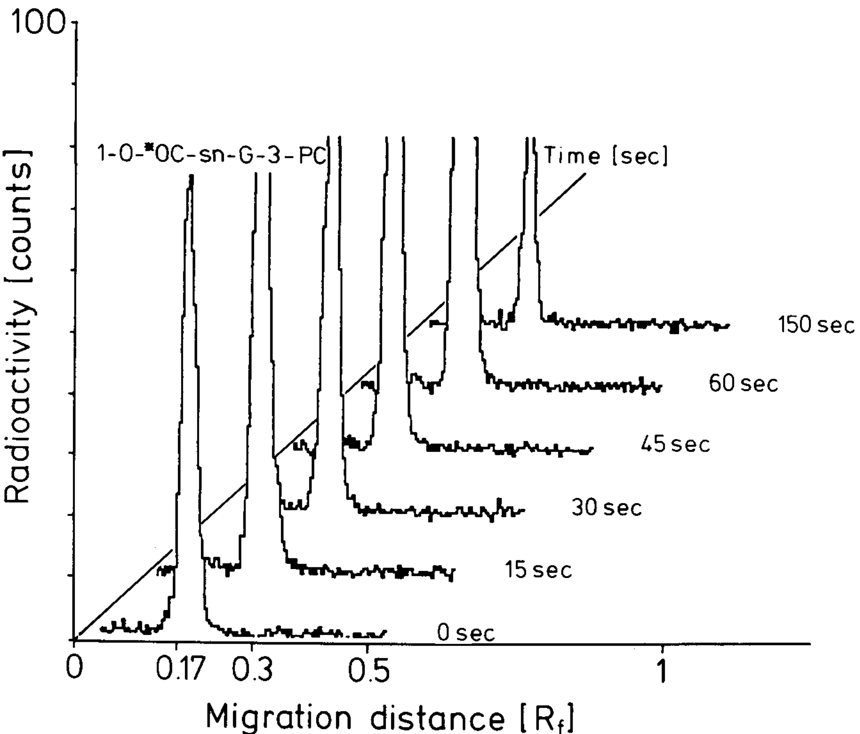

FIG. 2. Acylation of 1-*O*-[^{3}H]octadecyl-lysolecithin with [1-^{14}C]oleoyl-CoA. Microsomal protein (0.15 mg) was incubated with 170 nmol 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (1-O-*Oc-*sn*-G-3-PC) and 50 nmol [1-^{14}C]oleoyl-CoA for 0, 15, 30, 45, 60 and 150 sec in an incubation volume of 1 ml. The reaction was stopped by formic acid, and the lipids were extracted and identified as described in Figure 1. No products were formed in the indicated time period. In addition, we performed incubations for longer time periods. Even after 5 and 10 min, no product formation was detected. Therefore, the specific rate of the reaction, if any, is less than 60 pmol/mg protein/hr. This demonstrates that acyl-CoA-dependent acyltransferases cannot acylate ether lysophospholipids.

reaction was stopped with 100 µl of 60% formic acid. Extraction of lipids was performed as described for the acylation assay.

RESULTS AND DISCUSSION

Lysophospholipids have been described as potent antineoplastic agents (24–28). Therefore, it is important to understand the metabolic fate of these molecules in the living cell. In this study, we compare the properties of ester and ether lysophospholipids as substrates for acyltransferases, which catalyze the synthesis of phospholipids. These acyltransferases are important enzymes for detoxification of lysophospholipids. They have been described first by Lands (3) as acyl-CoA-dependent enzymes in rat liver and are mainly localized in membranes of the endoplasmatic reticulum, as shown by Eibl et al. (29). Their acyl-CoA requirement was applied to develop a spectrophotometric assay with DTNB as the sensitive indicator for the appearance of sulfhydryl groups (4). Since amounts of 1 µmol DTNB per incubation volume of 1 ml already inhibited the enzyme reaction, we used smaller amounts of DTNB and obtained satisfactory results with only 0.2 µmol DTNB (see Table 1). The inhibitory effect of DTNB was more pronounced for the substrate combination lyso-PC/oleoyl-CoA, which showed a decrease in specific activity of about 20%, than for the combination lyso-PA/oleoyl-CoA, which showed a drop of only 11%. The effect on the hydrolase activity was large, and the decrease of specific activity amounted to about 50%, however, on a much lower level of total activity as for the transfer rates.

The results obtained in the optimized spectrophotometric assay are summarized in Table 2. High incorporation rates for oleic acid are observed with lyso-PC and lyso-PA as fatty acid acceptors. The rates are lower for lyso-PE and the respective lyso-PS and lyso-PI. The acylation rates for arachidonoyl-CoA with the different lysophospholipids are quite similar in comparison to the incorporation of oleic acid. The specific rate is high for lyso-PC and the rates are low for lyso-PE and lyso-PS. However, there is a large difference in the acylation rates for lyso-PA (de novo synthesis) and lyso-PI (deacylation-reacylation cycle). For lyso-PA, incorporation of oleic acid is high and acylation with arachidonic acid is very low; vice versa for lyso-PI as substrate: the incorporation of oleic acid is low and the acylation with arachidonic acid compares with the high rates measured for the incorporation into lyso-PC. This is in agreement with the generally accepted finding that biosynthetically formed phosphatidic acid is low in arachidonic acid and that phosphatidylinositol is relatively high in it. No detectable transfer was observed with alkyl lyso-PC (<5 nmol/min/mg protein), which may explain the higher toxicity of this

compound in different cell lines in comparison to the ester analog.

The spectrophotometric test is not sensitive enough to measure very low incorporation rates (<5 nmol/min/mg protein). Since the DTNB-test reports the appearance of free sulfhydryl groups, it measures the sum of two enzyme activities: the acyltransfer and the hydrolase reaction. If acyltransfer and hydrolase rates are in the same magnitude order, the calculated acyltransfer rates become unreliable. In this case, a direct measurement of the products formed in the current reaction is necessary; this requires radiolabeled substrates.

In the first set of experiments, shown in Figure 1, the acyltransfer rate for 1-*O*-[^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine/[^{14}C]oleoyl-CoA was determined by following the formation of 1-*O*-[^{14}C]palmitoyl-2-*O*-[^{14}C]oleoyl-*sn*-glycero-3-phosphocholine (Fig. 1). The specific activity for this reaction was 172 nmol/min/mg protein, which is much higher than 134 nmol/min/mg protein, the rate observed in the optimized spectrophotometric assay. It again demonstrates that even low concentrations of DTNB interfere with the acylation reaction. While the spectrophotometric assay does allow comparison of acyltransferase activities, absolute rates of enzyme activity need to be determined in the absence of DTNB.

In the second set of the radioactive incubations, we used 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine/[^{14}C]-oleoyl-CoA as substrates. No product was formed in the indicated time period (Fig. 2). The sensitivity of the test does allow the identification of 100 dpm (0.5 cpm), which corresponds to 1 pmol product. Since we were not able to detect any product after 10 min of incubation (data not shown), the rate of acylation, if any, is less than 60 pmol/mg/hr. This indicates that an exchange of an ester for an ether group in lysolecithin results in what seems a complete loss of substrate properties for the acyl-CoA-dependent acyltransferases from rat liver microsomes. So, 1-acyl lysolecithins are substrates for the acyltransferases, whereas 1-alkyl lysolecithins are not.

In recent publications by Kramer et al. (17,30), it was described that an acyl-CoA-independent pathway for the acylation of ether lysophospholipids exists in human platelets. It requires 1,2-diacyl-*sn*-glycero-3-phospholipids as a cofactor. We searched for a similar reaction in rat liver. Using 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine as an acyl acceptor and 1-*O*-stearoyl-2-*O*-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphoinositol as acyl-donor in incubations with rat liver microsomes, we observed the formation of 1-*O*-[^{3}H]-octadecyl-2-*O*-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine (Fig. 3). This product contains both labels, ^{3}H and ^{14}C, in a ratio of 1:1 on a molecular basis. A rate of 42 nmol/mg protein/hr was calculated for the CoA-independent transacylation reaction. This rate is much higher than the estimated maximum rate for the acyl-CoA-dependent acyltransferase reaction (60 pmol/mg protein/hr, based on the sensitivity limit of the test). Therefore, we conclude that the acyl-CoA-independent transfer reaction is the dominant pathway for the acylation of ether lysolecithins in rat liver.

From these experimental results of the acylation of ester and ether lysophospholipids, we conclude that two independent pathways of acylation exist in rat liver. The first route depends on acyl-CoA as cosubstrate and is dominant for ester lysophospholipids; the second route

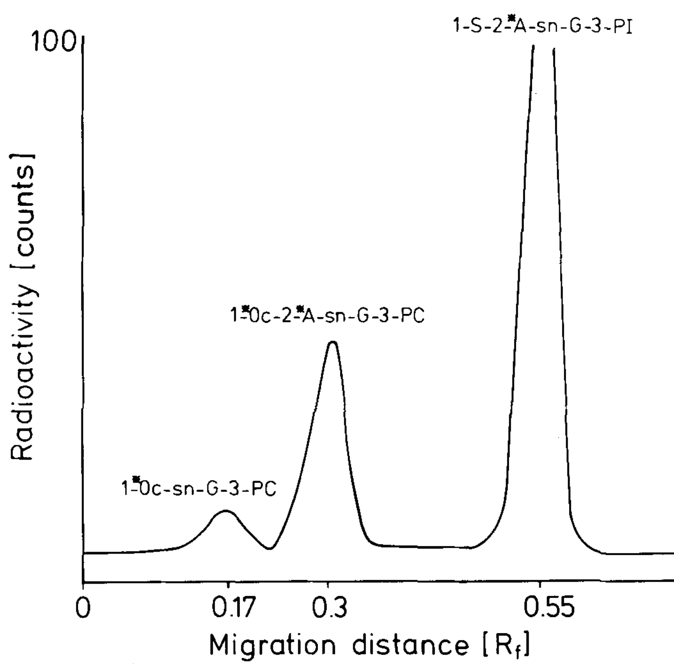

FIG. 3. Acylation of 1-*O*-[^{3}H]octadecyl-lysolecithin with 1-*O*-stearoyl-2-*O*-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoinositol as a fatty acid donor (transacylation). 1-*O*-Stearoyl-2-*O*-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphoinositol (1-S-2-*A-*sn*-G-3-PI) (8 nmol) and 1-*O*-[^{3}H]octadecyl-*sn*-glycero-3-phosphocholine (1-*Oc-*sn*-G-3-PC) (10 nmol) were incubated with 0.15 mg microsomal protein in an incubation volume of 1 ml. After 60 min, the reaction was stopped with formic acid, and the lipids were extracted and identified as described in Figure 1. The radioactive reaction product was identified as 1-*O*-octadecyl-2-*O*-arachidonoyl-phosphocholine (1-*Oc-2-*A-*sn*-G-3-PC). Counts in the figure represents both ^{14}C- and ^{3}H-activity after 10 min. The product of the reaction in the middle peak contains ^{14}C and ^{3}H activity. This peak's area was collected, and ^{14}C- and ^{3}H-activities were measured separately in a Zinser III Liquid Counter. The ratio of ^{14}C/^{3}H was close to 1, as expected. Control runs with both starting materials did not show radioactivity in the area of the enzyme reaction product.

is independent from acyl-CoA and uses diacyl phospholipids as a fatty acid source. This pathway is dominant for the acylation of ether lysophospholipids with arachidonic acid.

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Increased Membrane Permeability for an Antitumoral Alkyl Lysophospholipid in Sensitive Tumor Cells[1]

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We have investigated cellular sensitivity to the antitumoral alkyl lysophospholipid (ALP) 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($ET\text{-}18\text{-}OCH_3$) in vitro. The permeation of this lipid into the cell was not influenced by metabolic inhibitors of ATP biosynthesis. $ET\text{-}18\text{-}OCH_3$ uptake was not saturable within sublytic concentrations, but could be inhibited in part by cytochalasin B (CB) and dipyridamole. The activation energy of the CB-sensitive uptake process was increased up to threefold compared to CB-insensitive uptake. $ET\text{-}18\text{-}OCH_3$ influx and equilibrium binding of $ET\text{-}18\text{-}OCH_3$ were decreased in a fibrosarcoma cell variant (MethA) selected for $ET\text{-}18\text{-}OCH_3$ resistance. The resistant MethA cells were also less sensitive to cytolysis by lysophosphatidylcholine and other ALP. After 72 hr, the resistant MethA cells had metabolized only 11.8% more of the absorbed $ET\text{-}18\text{-}OCH_3$ than sensitive MethA cells. However, they tolerated at least a 30-fold concentration of this ALP. The uptake mechanism, which could be inhibited by CB, was less active in resistant MethA cells and several other ALP-resistant cell lines. The concentration of CB, required for maximal uptake inhibition, was increased more than four times in the ALP-sensitive tumor cell lines. CB-specific $ET\text{-}18\text{-}OCH_3$ uptake was also enhanced after virus transformation of 3T3 fibroblasts by SV 40. Dipyridamole retarded the $ET\text{-}18\text{-}OCH_3$-mediated cell destruction.
Lipids 22, **813–819 (1987).**

Alkyl lysophospholipids (ALP) are stable ether or thioether analogues of 2-lysophosphatidylcholine (2-LPC), which is present in 3–12% of the total phospholipids in cellular membranes and plasma (1). ALP cannot enter the deacylation-acylation cycle of cellular phospholipids (2) because a lysophospholipase cannot split the alkyl bond, and acylation is blocked in most of these compounds by a stable substitution of position *sn*-2 of the glycerol backbone.

Among many biological effects, ALP inhibit growth and metastasis of different syngeneic tumors in the mouse (for review, see ref. 3). Moreover, the results of a clinical phase I study suggest that ALP might be useful in human cancer therapy (4). The antineoplastic activity is probably caused by the generation of tumoricidal macrophages (5) and a direct progressive destruction of tumor cells, whereas nontransformed cells are less affected (5–7). Some evidence has been reported (6–10) suggesting that sensitive cells take up more ALP or fail to split the ether bond because of the lack of a glyceryl-ether cleaving enzyme (E.C. 1.14.16.5). One of the most potent ether analogues of lysolecithin that we tested was the 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($ET\text{-}18\text{-}OCH_3$). Here we report on cellular metabolism and cellular uptake mechanism of this compound in sensitive and resistant cell lines.

MATERIALS AND METHODS

Alkyl lysophospholipids. 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (L-ET-16-OH), 3-*O*-hexadecyl-*sn*-glycero-1-phosphocholine (D-ET-16-OH) and 1-*O*-octadecyl-9′-enyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine were purchased from R. Berchtold (Biochemical Laboratory, Bern, Switzerland).

1-*O*-octa-[9,10(n)-^{3}H]decyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($ET\text{-}18\text{-}OCH_3$) was obtained by catalytic reduction of 1-*O*-octadecyl-9′-enyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine with tritium gas (Amersham Radiochemical Center, Buckinghamshire, U.K.). The specific activity was 3.53 Ci/mmol. Unlabeled $ET\text{-}18\text{-}OCH_3$ was purchased from Medmark Chemicals (Gruenwald, Muenchen, FRG).

Other chemicals. Cytochalasin B (CB), cytochalasin E (CE), dipyridamole (DP), papaverine and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and sodium pyruvate were purchased from Gibco (Karlsruhe, FRG). FBS was used after inactivation for 1 hr at 50 C. 2-LPC was purchased from Roth (Karlsruhe, FRG).

Cells and cell culture. The cell lines listed in Table 1 were cultivated with DMEM plus 10% fetal bovine serum (FBS; v/v), 1 mM sodium pyruvate, 5×10^{-5} M β-mercaptoethanol, 50 units penicillin and 50 μg streptomycin per ml at 37 C in a humidified 10% CO_2 atmosphere. All cells were used in the log phase of growth. Viability, tested by trypan blue dye exclusion, was never below 90%.

(BALB/c $\times$ C57BL)F_1 bone marrow–derived macrophages (MO) were cultivated in Teflon bags, as previously described (6). The cultures usually reached confluence after 12 days. The drug-resistant fibrosarcoma cells were

TABLE 1

Cell Lines Used in This Study

Abbreviation in text	Cell type (reference)
MethA	Methylcholanthren-induced fibrosarcoma (11)
rMethA	ALP-resistant variant of MethA (—)
P815	Methylcholanthren-induced mastocytoma (12)
Abels.	Abelson lymphoma (13)
YAC	Molony virus-induced murine lymphoma (14)
SV40-3T3	Simian virus 40 transformed 3T3 fibroblast (15)
3T3	Fibroblast of a Swiss mouse embryo (16)
L929s	Fibroblast from connective tissue (17)

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Abbreviations: ALP, alkyl lysophospholipids; 2-LPC, lysophosphatidylcholine (1-acyl-*sn*-glycero-3-phosphocholine); $ET\text{-}18\text{-}OCH_3$, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; D-ET-16-OH, 3-*O*-hexadecyl-*sn*-glycero-1-phosphocholine; L-ET-16-OH, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; CB, cytochalasin B; CE, cytochalasin E; DP, dipyridamole; FBS, fetal bovine serum; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; MO, macrophage; PBS, phosphate-buffered saline.

derived from surviving cells of cultures, which had been propagated in the presence of 6 μg ET-18-OCH_3/ml. These cells were further selected by cultivation with succeeding 10, 20 and 30 μg/ml of ET-18-OCH_3. Before use, resistant fibrosarcoma variant (rMethA) cells were propagated for several days in culture medium without this ALP and washed repeatedly. This did not alter the induced resistance to ET-18-OCH_3. 3T3 Fibroblasts were used in a nonconfluent state (3–4 × 10^5 cells per cm^2). For absorption studies, 3T3, SV40-3T3 and L929s fibroblasts were harvested by treatment with a 0.25% solution of trypsin (Boehringer, Mannheim, FRG) for 10 min. Trypsin was inactivated by FBS, and cells were washed twice before use. This procedure had no influence on ET-18-OCH_3-uptake.

Lipid extraction and lipid analysis were performed as previously described (6). In brief, lipids were extracted as described by Ways and Hanahan (18), separated on silica-coated thin layer plates using chloroform/methanol/H_2O (60:40:10, v/v/v) as solvent. The separated compounds were detected by iodine vapor and scraped off into scintillation vials. One ml of H_2O was added, and the vials were shaken for 24 hr to obtain a very fine suspension. The samples were measured in a scintillation counter after adding 12 ml of Bioflur (New England Nuclear, Boston, MA).

Binding of ET-18-OCH_3 to serum components. For separation of lipoproteins, we used a previously described method (19) with a few modifications. FBS was brought to a density of 1.21 g/cm^3 by addition of solid NaBr. The samples were allowed to equilibrate for 15 min at 37 C in the presence of ^{3}H-labeled ET-18-OCH_3 (5 μg/ml). Each 1.25 ml of the samples was pipetted into a 11 × 60 mm polyallomer centrifuge tube (Beckman Instruments, Palo Alto, CA). A discontinuous gradient was formed by carefully layering 1 ml of NaBr solution with a density of 1.063 g/cm^3 above the serum, followed by 1 ml with a density of 1.019 g/cm^3 and finally 1 ml with a density of 1.006 g/cm^3. To minimize mixing, salt solutions were allowed to gravity-feed down the side of the tubes through a glass capillary. Six samples were centrifuged at 20 C for 24 hr at 400,000 × g in an SW-56 rotor (Beckman). Each 0.25 ml of the samples was succeedingly measured by liquid scintillation counting after the addition of 12 ml Bioflur.

Absorption and inhibitor studies. CB, CE and DP were stored in a stock solution with dimethyl sulfoxide (DMSO) at −20 C. If not otherwise indicated, 10^6 cells were preincubated for 1 hr with 1 ml of DMEM containing 10% FBS, 0.5% DMSO and the inhibitor. We incubated the samples at 37 C in a humidified 10% CO_2 atmosphere for another 2 hr with labeled ET-18-OCH_3 (1 μg/ml). Absorption was stopped by addition of 10 ml of an ice-cold, phosphate-buffered NaCl-solution (0.9%, pH 7.2; phosphate buffered saline [PBS]). Samples were immediately centrifuged and washed twice with 10 ml of chilled PBS. No significant loss of absorbed ET-18-OCH_3 occurred by this procedure, since transport activity was minimal at this temperature. A portion of the cell suspension was resolved in Bioflur (New England Nuclear) and assayed for liquid scintillation counting.

When the temperature dependence of ET-18-OCH_3 uptake was determined, cells were incubated with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–buffered DMEM containing 10% FBS in a thermostated water bath. After the temperature had equilibrated, absorption of ET-18-OCH_3 (2 μg/ml) within 1 hr was measured as described above. When the membrane translocation of ET-18-OCH_3 was determined, we used the previously described albumin-extraction method (20). Therefore, cells were washed twice with 10 ml chilled PBS containing 1% fatty acid-free BSA.

Assays for the cytotoxicity of ET-18-OCH_3. 5 × 10^5 Cells/ml DMEM plus 10% FBS were cultivated with the indicated concentration of ET-18-OCH_3 for 24 hr. Cells were washed once, and ^{3}H-thymidine incorporation was measured as described (6). When the influence of DP on ET-18-OCH_3 cytotoxicity was studied, cells were preincubated without ET-18-OCH_3 for 4 hr in the presence or absence of DP, which was added in an ethanol solution. The final ethanol concentration was 0.05%. MethA cells (5 × 10^4/ml) were incubated for 72 hr and washed twice before ^{3}H-thymidine incorporation was measured.

Since mature bone marrow MO do not incorporate thymidine, the number of viable cells was determined with a culture counter after 72 hr of incubation. Therefore, 2 × 10^6 cells were placed into culture dishes with a hydrophilic Teflon membrane (Petriperm, Heraeus, FRG). The final volume was 5 ml (DMEM plus 10% FBS). Under these conditions, viable cells remained attached on the surface of the dishes, whereas damaged cells detached and could be removed by washing the monolayers once with PBS. The number of nonadherent but not trypan blue–stained cells was always below 8% of control cultures. The adherent cells were then harvested with a rubber policeman. When ALP-induced cytolysis in the absence of FBS was determined, 10^6 cells/ml were incubated with 7.5 μg ALP (2-LPC) per ml of DMEM for 30 min in a 10% CO_2 atmosphere at 37 C. The percent of trypan blue–dyed cells was immediately determined by counting 200 to 300 cells with a haematocytometer.

If not otherwise indicated, all experiments were done in triplicate and standard deviations were below 10%.

RESULTS

Selective cytotoxicity and cellular metabolism of ET-18-OCH_3. Already after 24 hr of incubation, differences in ET-18-OCH_3 resistance are revealed (Fig. 1). 3T3 and L929s fibroblasts were less sensitive, but 48 hr later only the 3T3 cells survived a concentration of 20 μg/ml ET-18-OCH_3, and ^{3}H-thymidine incorporation remained at 80% of control. This value decreased to less than 10% of control with L929s fibroblasts after 72 hr. Abels., P815, SV40-3T3 and MethA were the most sensitive tumor cell lines. Bone marrow–derived MO were only moderately resistant against ET-18-OCH_3 (see Fig. 7). In contrast, rMethA could be cultivated continuously in the presence of 30 μg/ml ET-18-OCH_3 with a doubling time of 20–24 hr.

The metabolism of ET-18-OCH_3 was low in all cell lines studied. After 12 hr of incubation, between 88.9 and 94.6% of the absorbed compound was unchanged. Even after 72 hr, only 8.4% in MethA and 20.2% in rMethA were metabolized. On thin layer chromatography, the ET-18-OCH_3 metabolites migrated with oleic acid, neutral lipids, phosphatidylethanolamine and phosphatidylcholine. We did not further analyze these metabolites. At present, it is not completely clear whether ALP

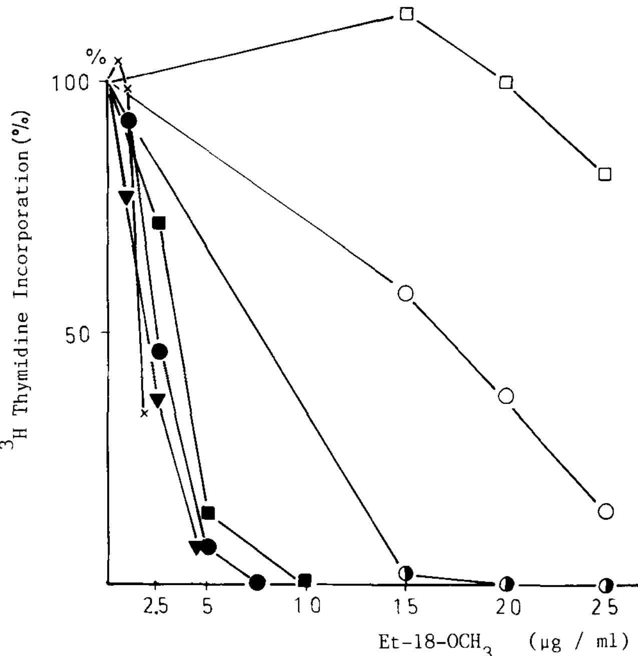


FIG. 1. ET-18-OCH$_3$-induced growth inhibition. 5 × 10^5 Cells/ml DMEM plus 10% FBS were cultivated for 24 hr with the indicated concentration of ET-18-OCH$_3$. Cells were washed once and ^{3}H-thymidine incorporation within 4 hr was determined. Mean of 4 separate determinations; SD ⩽ 15%. □, 3T3; ○, L929s; ◑, YAC; ■, SV40-3T3; ●, MethA; ▼, P815; x, Abels.

cytotoxicity is primarily due to direct lysis by the accumulated compound itself or to secondary toxic effects induced by ALP, like a disturbance of lipid metabolism (6). Table 2 shows that drug resistance in rMethA was accompanied by increased cell membrane stability to cytolysis by different ALP and 2-LPC.

Uptake and equilibrium binding of ET-18-OCH$_3$. Figure 2 shows the time course of ET-18-OCH$_3$-uptake in the presence or absence of FBS (10% v/v). Within the first 2 hr, the rate of uptake was enhanced about twofold in the sensitive cells. It should be noted that rMethA cells had been cultivated several days without ET-18-OCH$_3$ and washed repeatedly. Since ET-18-OCH$_3$ uptake was reduced in both cell variants in the presence of serum, we examined the binding of ET-18-OCH$_3$ to serum components. After density gradient separation of FBS, more than 90% of the added ET-18-OCH$_3$ was bound to a serum fraction with a density >1.21 g/cm^3, which has been previously identified as albumin (21). This indicates that ET-18-OCH$_3$ has the same high affinity binding to serum albumin as also described for natural LPC (21). In the presence of serum, the uptake rate was about linear with respect to time up to 4 hr (Fig. 2), and further studies were done within this period.

After 2 hr of incubation and two saline washes, only 10.3% of the MethA-bound ET-18-OCH$_3$ could be extracted by two washes with a chilled solution of 1% BSA in PBS. It has been demonstrated previously (20) that albumin washing removes the LPC from the outer cell membrane monolayer because albumin binds LPC with high affinity. Since ET-18-OCH$_3$ had the same high affinity to albumin like LPC, the albumin-unextractable ET-18-OCH$_3$ in MethA cells was probably inaccessible to albumin and was therefore not located on the cell surface. This finding is supported by the observation that equilibrium establishment between albumin-bound and cell surface-bound LPC is rapidly achieved, at least before 2 hr (20). In contrast, membrane translocation of LPC (20) or cellular uptake of ET-18-OCH$_3$ in the presence of serum (Fig. 2) is a comparatively slow process, i.e., the equilibrium time for cellular ALP uptake is 20–24 hr. Albumin-unextractable ET-18-OCH$_3$ uptake (influx) was increased 2.7 times in sensitive MethA. After equilibrium

TABLE 2

ALP-Induced Cytolysis in MethA and Drug-Resistant Mutant Cells (rMethA)

Compound	Increase in trypan blue-dyed cells (% of total cell number) MethA	rMethA
2-LPC	23 (±6)	5 (±1)
D-ET-16-OH	87 (±4)	29 (±6)
L-ET-16-OH	70 (±2)	8 (±2)
ET-18-OCH$_3$	57 (±3)	31 (±3)

10^6 Cells/ml were incubated for 30 min at 37 C in the presence of 7.5 µg/ml compounds in the absence of fetal bovine serum. Two hundred to 300 cells were counted for determinations of the percent of trypan-blue dyed cells. Mean of three separate experiments (± SD).

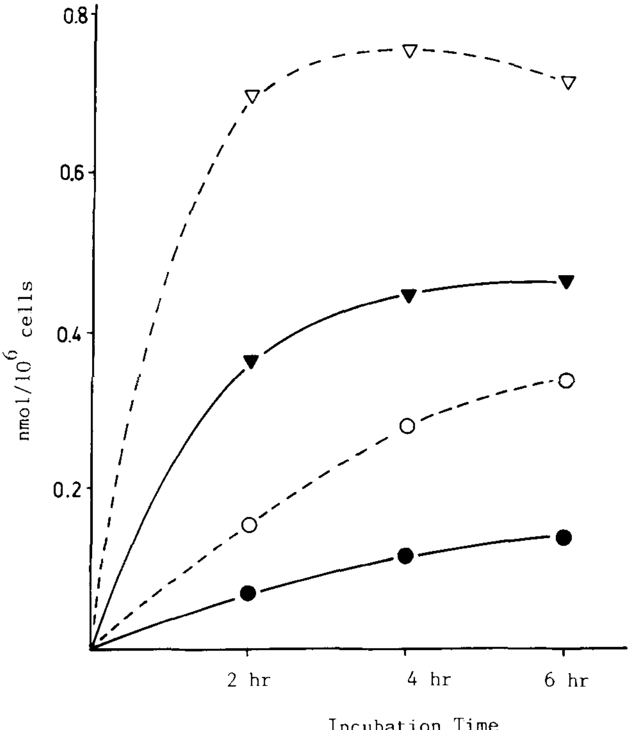


FIG. 2. Time course of ET-18-OCH$_3$ absorption in the presence and absence of FBS. 10^6 Cells/ml were incubated at 37 C with 1 µg/ml ET-18-OCH$_3$ in the presence or absence of 10% (v/v) FBS. Absorption was measured as described in Materials and Methods. ○, MethA plus FBS; ▽, MethA without FBS; ●, rMethA plus FBS; ▼, rMethA without FBS.

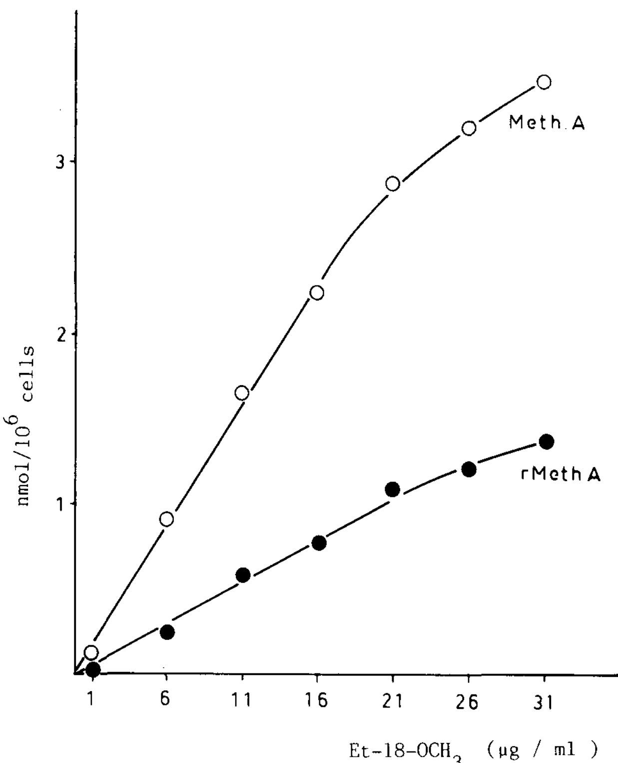


FIG. 3. Concentration dependence of ET-18-OCH_3-uptake in MethA and rMethA cells. 10^6 Cells/ml DMEM/10% FBS were incubated with the indicated concentration of ET-18-OCH_3 for 2.5 hr. Absorption was measured as described in Materials and Methods.

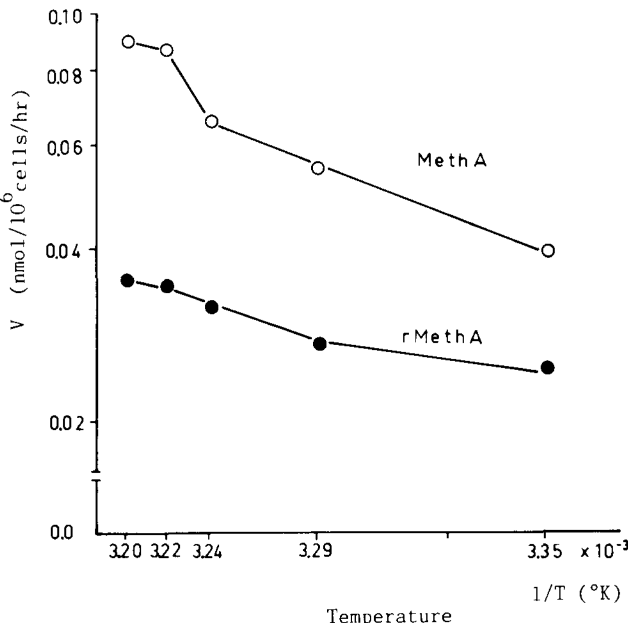


FIG. 4. Arrhenius plots for the uptake of ET-18-OCH_3 by normal and resistant MethA cells. 0.5 × 10^6 Cells/ml DMEM/10% FBS were incubated at the indicated temperature for 1 hr with 2 μg/ml ET-18-OCH_3. Uptake was determined as described in Materials and Methods. Mean of 3 experiments, SD ≤ 5%.

establishment, sensitive MethA cells bound 3–4 times as much of the drug as did the resistant mutant cells, indicating that, besides enhanced influx, the number of cellular binding sites was also increased in the sensitive cells.

Temperature and concentration dependence of ET-18-OCH_3-uptake. Figure 3 shows that uptake of ET-18-OCH_3 is about linear with respect to extracellular concentration up to 20 μg/ml. Only a slight saturation was visible above this concentration in MethA and rMethA.

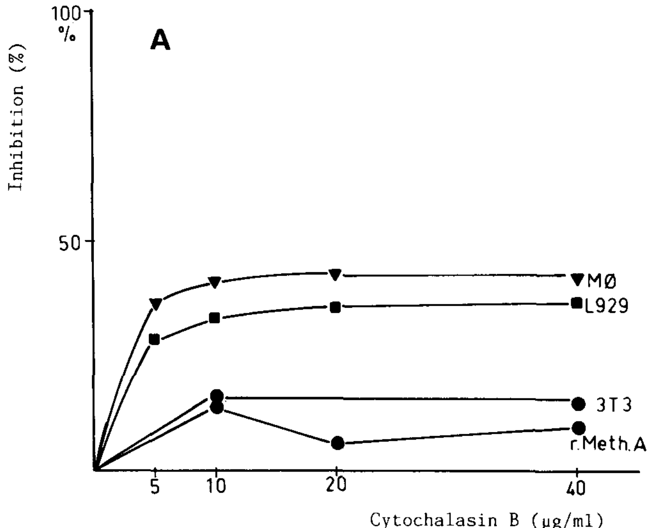
Figure 4 shows the temperature dependence of ET-18-OCH_3 uptake. The decrease in absorption rate at low temperature is probably caused by a decrease in lipid fluidity. The Arrhenius plots for MethA and rMethA reveal differences in the activation energy, which is about three times increased in the sensitive cells between 3.22 × 10^{-3} K (37 C) and 3.24 × 10^{-3} K (35 C) and below 3.29 × 10^{-3} K (31 C). At 0–4 C, both cell variants bound the same low amount of ET-18-OCH_3, which came close to 20% with respect to MethA at 37 C. In this case, 80% of the cell-bound ET-18-OCH_3 was extracted by albumin washing and was therefore mostly located on the cell surface.

This blocking of ET-18-OCH_3 transport into the cell is in agreement with the blocking of LPC transport at 0 C (20). Our results can be explained by two different uptake mechanisms in MethA and rMethA cells. In support of this, significant discontinuities in the Arrhenius plot are only seen with MethA cells at 3.24 × 10^{-3} K (37 C) and 3.22 × 10^{-3} K (35 C). A different membrane protein-lipid interaction must have influenced ET-18-OCH_3 uptake in these sensitive cells, and it could reflect a lipid-phase transition or a phase separation of membrane proteins involved in ALP transport.

Inhibitor studies. When we incubated the cells with 10^{-2} M NaN_3 plus 5 × 10^{-2} M 2-deoxyglucose or with 10^{-2} M NaF plus 10^{-2} M NaN_3, ET-18-OCH_3 uptake was inhibited for less than 10% in MethA and rMethA. Similar effects were seen with macrophages and L929s fibroblasts. It has been demonstrated previously that the cellular ATP level drops rapidly after this treatment, and endocytosis is also blocked (22). Therefore, the ET-18-OCH_3 uptake in our studies did not occur via endocytosis, and it depended apparently not on cellular energy.

Many nonsaturable transport processes are inhibited by DP, CB and papaverine (23). ET-18-OCH_3 uptake into sensitive MethA cells could also be inhibited by these compounds, but uptake into rMethA was hardly affected. Both cell variants had about the same uptake rates in the presence of 40 μg/ml CB (Table 3).

Figures 5A and 5B show the influence of CB concentration on the inhibition of ET-18-OCH_3 uptake. In 3T3, MO, L929s and rMethA, maximal inhibition occurred at 5–10 μg/ml CB. In contrast, with Abels., P815, YAC, SV-40-3T3 and MethA, more than 40 μg/ml CB was necessary to complete uptake inhibition (Fig. 5B). Figure 5C shows the inhibition of ET-18-OCH_3 uptake by DP (this drug had a limited solubility of about 20 μg/ml under our incubation conditions, pH 7.4). DP was as effective as CB. Papaverine also acted as an inhibitor, but CE had hardly any effect (results not shown). The inhibition of ET-18-OCH_3 uptake by CB did not change at a molar ratio of ET-18-OCH_3 to CB between 0.1 and 3.1. This indicates that uptake inhibition was not caused by the formation of an ET-18-OCH_3–CB complex and that CB is no competitive inhibitor of ET-18-OCH_3 uptake. Table 3 shows the amount of ET-18-OCH_3 absorbed in the presence



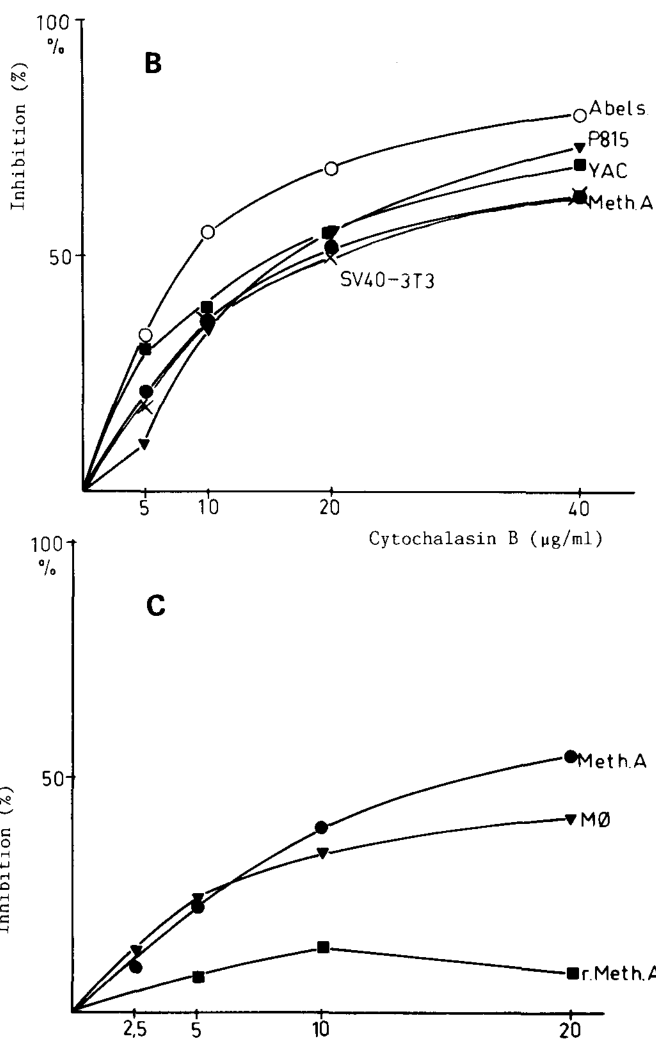


FIG. 5. Inhibition of ET-18-OCH_3-uptake by CB and DP. 10^6 Cells/ml DMEM/10% FBS were first incubated for 1 hr with the indicated concentration of the inhibitor. Then ET-18-OCH_3 was added and uptake determined after another 2 hr, as described in Materials and Methods. A shows the more resistant cell lines, where inhibition was complete at about 10 µg/ml CB. B shows the sensitive cell lines, where maximal inhibition was not achieved even with 40 µg/ml CB. C shows the inhibition of ET-18-OCH_3-uptake by dipyridamole.

TABLE 3

Uptake of ET-18-OCH_3 in the Presence or Absence of Cytochalasin B (40 µg/ml)

	ET-18-OCH_3 (pmol/10^6 cells)		
Cell line	−Cytochalasin B	+Cytochalasin B	Cytochalasin B-specific uptake
MO	129 ± 5	75 ± 2	54
MethA	158 ± 3	77 ± 1	≥81
rMethA	83 ± 3	76 ± 2	7
3T3	109 ± 1	94 ± 8	15
SV40-3T3	146 ± 9	53 ± 4	≥93
L929s	72 ± 4	46 ± 0.7	26
Abels.	116 ± 4	23 ± 0.7	≥93
P815	222 ± 4	60 ± 2	≥162
YAC	82 ± 3	25 ± 1	≥57

10^6 Cells/ml DMEM/10% FBS were incubated for 2 hr in the presence or absence of 40 µg/ml cytochalasin B (CB), which was added in a DMSO solution (final DMSO concentration in all samples 0.5%). Uptake of ET-18-OCH_3 was determined as described in Materials and Methods. CB-specific ET-18-OCH_3 uptake was calculated from the difference of both values if inhibition seemed complete at 5–10 µg/ml CB (see also Figs. 5A and 5B). If maximal inhibition was not achieved at 40 µg/ml CB, it is indicated that CB-specific uptake is even higher. Mean of three separate determinations ± SD.

or absence of 40 µg/ml CB. From these data, the CB-dependent ET-18-OCH_3-uptake was calculated. Only the CB-inhibitable (=CB-specific) uptake was decreased in the resistant variant of MethA. The most sensitive cell lines (Abels., MethA, P815 and SV40-3T3) showed the highest activities of this CB-dependent ALP transport system.

Influence of DP on cytotoxicity of ET-18-OCH_3. The lower activity of a CB-specific transport system was a characteristic of ET-18-OCH_3 resistance. Thus it should be possible to render cells more resistant to ET-18-OCH_3 by inhibition of this uptake process.

This was demonstrated by addition of 5–10 µg/ml DP together with ET-18-OCH_3 to the cell cultures. Figures 6 and 7 show that DP protected MethA and MO in a concentration-dependent manner. Cell destruction of MO was retarded to a greater extent. We had assured ourselves that the number of living cells correlated to ^3H-thymidine incorporation under these conditions.

DISCUSSION

It was shown in this study that various cell lines exhibit a very different sensitivity to the antitumoral ALP ET-18-OCH_3 in vitro. A highly drug-resistant variant of a methylcholanthren-induced fibrosarcoma cell line (MethA) was isolated. We used these cells to study the mechanism of ALP resistance. In accordance with Hoffman et al. (24), we found that metabolism of ET-18-OCH_3 was negligible, but cellular drug concentration after equilibrium binding was 3–4 times decreased in the resistant cells. Our results indicate that this was caused by a lower influx together with a decreased number of cellular binding sites. Since cell mass of MethA and rMethA was equal, ET-18-OCH_3 was probably not equally distributed over the cell but stored in distinct cellular structures. Another indication for a nonhomogenous

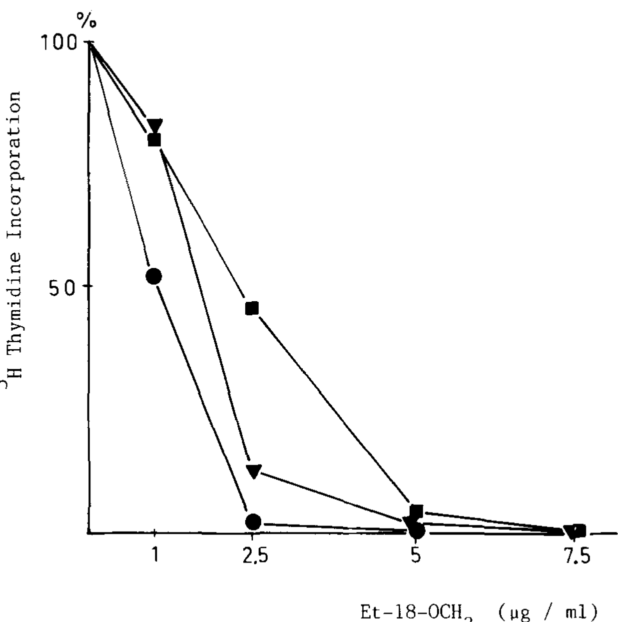


FIG. 6. Influence of DP on the ET-18-OCH$_3$-mediated cytotoxicity for MethA cells. 5 × 10^5 Cells/ml DMEM/10% FBS were incubated with the indicated concentration of ET-18-OCH$_3$ for 72 hr. Since DP had to be added in an ethanol solution, all samples were adjusted to the final ethanol concentration of 0.05%. Before the determination of ^{3}H-thymidine incorporation, cells were washed twice. Mean of 2–3 independent experiments, done with triplicate cultures, SD ⩽ 10%. ●, Control; ▼, 5 µg/ml DP; ■, 10 µg/ml DP.

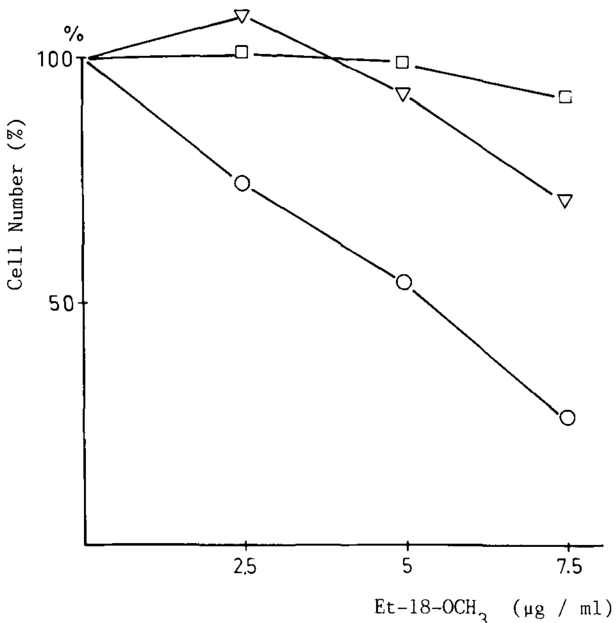


FIG. 7. Influence of DP on the ET-18-OCH$_3$-mediated cytotoxicity for bone marrow-derived macrophages. 2 × 10^6 Cells/5 ml DMEM/10% FBS were cultivated for 72 hr. DP was added in an ethanol solution (final ethanol concentration in all samples was 0.05%). The number of living cells was determined as described in Materials and Methods. Each point is the mean of two independent experiments done with triplicate cultures, SD ⩽ 15%. ○, Control; ▽, 5 µg/ml DP; □, 10 µg/ml DP.

distribution of ALP was recently described (24). The resistant mutant cells also showed a substantially lower sensitivity to direct lysis by different lysophospholipids. These findings suggest that ALP-binding sites within the cell membrane could also be responsible for ALP-induced cell lysis. Moreover, the clustering or an increased number of ALP-binding sites could explain the selectivity of ALP-induced inhibition of membrane-bound enzymes in some tumor cells (6,25,26). Local accumulation of ALP within the cell membrane could also increase the permeability for ions like Ca (27) and thereby cause tumor growth inhibition. Uptake of ET-18-OCH$_3$ was not saturable within sublytic concentrations of the drug and, moreover, not influenced by inhibitors of ATP biosynthesis. Because of the cytolytic activities of the ALP (28), however, it cannot be excluded that uptake saturation would appear at higher concentrations. Our results with a previously published albumin extraction method (20) strongly suggest that ET-18-OCH$_3$ permeated across the cell membrane. Interestingly, CB inhibited this translocation specifically. It has been suggested that CB and other membrane-active compounds like DP, papaverine and so on may interact with hydrophobic regions of integral membrane proteins (29), because CB was not bound to membrane lipids (30) and had no influence on membrane lipid fluidity (31). Integral membrane proteins can act as specific transport sites by disturbing the order of the lipid bilayer. The different activation energy of normal and CB-inhibitable ET-18-OCH$_3$ uptake is probably a further indication for specialized membrane structures of ALP transport. CB could bind directly or in close proximity to ALP flip-sites and could sterically hinder the permeation of the drug. The lower sensitivity of several cell lines to the cytotoxicity of ET-18-OCH$_3$ corresponded to the decrease in CB-specific ET-18-OCH$_3$-uptake, but not to the overall ET-18-OCH$_3$-uptake (compare Fig. 1 to Table 3). We propose the hypothesis that ALP is transported across the lipid bilayer by a CB-specific integral membrane protein, which could also have an increased affinity to ALP. In the ALP-sensitive tumor cell lines—MethA, YAC, Abels. and P815—a higher concentration of CB was necessary to achieve maximal uptake inhibition. This was also observed after virus transformation of 3T3 fibroblasts. Since equilibrium binding studies have shown that SV40-3T3 and 3T3 cells have the same number of CB-binding sites per cell (31), ALP transport sites were probably only dislocated from CB-binding sites in SV40-3T3. This could be true for the other transformed cell lines.

The inhibition of ET-18-OCH$_3$-uptake by DP or CB showed similar characteristics. We could demonstrate that a partial inactivation of the transport system by DP rendered the cell more resistant to ET-18-OCH$_3$ cytotoxicity. Since DP is rather nontoxic, it could be used to protect a cell against ET-18-OCH$_3$, when damage is unfavorable. Moreover, it could be of advantage that MO are apparently protected to a greater extent than tumor cells like MethA. Maybe this is the result of a more effective inhibition of ALP-uptake into MO by DP (Fig. 5C). It should be noted that macrophages are important for the antitumoral activity of ALP in vivo (5).

A possible therapeutical value of the combined application of ALP and DP is now under study in experimental tumors in mice and rats.

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Accumulation of an Alkyl Lysophospholipid in Tumor Cell Membranes Affects Membrane Fluidity and Tumor Cell Invasion[1]

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Tumor cells grown in the presence of 1-*O*-alkyl-2-*O*-methylglycero-3-phosphocholine (AMG-PC) accumulated this ether lipid in their membranes. Depending on the cell type and the dose of the compound, up to 17% of the total phospholipids of the purified plasma membranes consisted of authentic AMG-PC. Extensive incorporation of the agent resulted in a decrease in plasma membrane fluidity and inhibition of tumor cell invasiveness in embryonic chick heart fragments. The extent of AMG-PC incorporation and fluidity change was not strictly correlated with the degree to which tumor cell invasion was inhibited.
Lipids 22, 820–823 (1987).

1-*O*-Alkyl-2-*O*-methylglycero-3-phosphocholine (AMG-PC) has been shown to inhibit specifically tumor cell growth and metastasis and is being tested as an anticancer drug in clinical trials (1). The mechanism of this drug's action is still unknown. In a previous report, we suggested a drug-induced alteration in membrane fluidity is possibly involved in the inhibition of tumor cell invasion in vitro (2). Here we have extended these investigations to four tumor cell lines, each of which differs in the degree of sensitivity to AMG-PC with respect to inhibition of cell growth and invasion. Incorporation of the drug was quantitated in subcellular fractions (an effort not previously undertaken) and was compared with the changes observed in membrane fluidity in these fractions. These dose-dependent alterations in the composition and structural order of membrane lipids were considered in relation to the degree of inhibition of tumor cell invasion in vitro.

MATERIALS AND METHODS

Cells and drug. MO_4 cells are virally transformed C_3H Mouse-fibroblastic cells that are invasive in vitro (2) and produce invasive and metastasizing fibrosarcomas in syngeneic mice (3). LLC-H61 is a highly metastatic subclone of the Lewis lung carcinoma cell line (4). The tumorigenic R1C cell line was established from baby rat kidney cells by transfection with a genomic fragment (map coordinates 0–16%) of the oncogenic adenovirus type 12 (5). Each of these adherently growing cell lines was cultured in Dulbecco's modified Eagle's medium (Flow Labs Ltd., Irvine, United Kingdom). HL-60 human promyeloid leukemia cells (6) were cultured in suspension in RPMI 1640 medium. The media were supplemented with 10% fetal calf serum and antibiotics (penicillin, 100 IU/ml; streptomycin, 50 μg/ml). The drug AMG-PC (racemic 1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine; ET-18-OCH_3, provided by W.E. Berdel, Technical University, Munich, Federal Republic of Germany) was dissolved in the culture media and added to the cell cultures 48 hr before harvesting the cells for the experiments.

Subcellular fractions. Cells were broken by pumping single-cell suspensions in Hanks' solution (Oxoid, London, United Kingdom) through an air-driven cell disruptor (Stansted Fluid Power Limited, Stansted, Essex, United Kingdom; model AO612, Disrupting Valve 516), using an air pressure of 45 lb/in^2 and a cell density of 5.10^7 cells/ml (7). A crude nuclear fraction was obtained by centrifugation twice at 1000×g for 10 min, with intermittent washing. Plasma membranes were purified from (1.95–945).10^4 g.min pellets, utilizing a discontinuous sucrose gradient, as described in detail (8). The purity of the plasma membrane preparations was ascertained routinely by electron microscopy and by marker assays, as described (8).

Lipid analysis. Lipids were extracted from the subcellular fractions with chloroform/methanol (2:1, v/v) followed by partition according to the method of Folch et al. (9). Phospholipids were separated by thin layer chromatography on precoated silica gel plates (E. Merck, Darmstadt, Federal Republic of Germany) using chloroform/methanol/0.5 M ammonia (50:65:9, v/v/v; two runs). AMG-PC and other phospholipids were quantitated by phosphate analysis (10).

In these experiments, we have also determined the amount of authentic AMG-PC accumulated in the two subcellular fractions (Table 2). The relative uptake of AMG-PC by the cells, as quantitated in these fractions, generally reflected the dose supplied in the culture medium. A high mol % of AMG-PC in the plasma membranes (MO_4, exp. 1; LLC-H61, exp. 2) was associated with an increase in the P_{DPH} value (Table 1). This was not the case in the nuclear fractions, a finding we do not clearly understand. This different effect of AMG-PC on the fluidity of plasma membranes and nuclei may relate to the different lipid composition of these two organelles, plasma membranes being typically enriched in cholesterol and sphingomyelin (ordering lipids; ref. 12). Alternatively, AMG-PC may have a metabolic effect, acting differently on the lipid composition (fluidity) of plasma and nuclear membranes. When expressed in mol % of the total phospholipids, the purified plasma membranes contained twice as much AMG-PC as the nuclei. When expressed as a ratio of AMG-PC to endogenous phosphatidylcholine, this factor was 3 to 4. This relative enrichment of AMG-PC in the plasma membranes may be even higher, when one considers a possible contamination of the nuclear fraction with plasma membranes.

In Table 3, the data are summarized and compared

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TABLE 1

Effect of AMG-PG on Membrane Fluidity of Various Tumor Cells and Subcellular Fractions, as Measured by Fluorescence Polarization with the Probe Diphenylhexatriene (P_{DPH} at 25 C)

			Fluorescence polarization (P_{DPH})		
Cells	Experiment	AMG-PC (μg/ml)	Intact cells	Crude nuclei	Plasma membranes (purified)
MO_4	1	—	0.281	0.265	0.332
		5	0.268	0.263	0.338
		10	0.260	0.268	0.346
	2	—	0.263	nd[a]	0.319
		10	0.254	nd	0.318
HL-60	1	—	0.248	0.220	0.291
		0.6	0.243	0.207	0.290
	2	—	0.199	0.188	0.256[b]
		0.75	0.188	0.179	0.250[b]
LLC-H61	1	—	0.280	nd	0.302
		10	0.284	nd	0.300
	2	—	0.257	0.246	0.293
		30	0.246	0.233	0.310
R1C		—	0.211	0.211	0.280
		5	0.214	0.214	0.302

[a]nd, Not determined.
[b]Measured in a crude membrane fraction (microsomes).

TABLE 2

Incorporation of AMG-PC in Tumor Cells as Quantitated in Purified Plasma Membranes and in Crude Cell Nuclei

	AMG-PC (mol% of total phospholipids)		AMG-PC/phosphatidylcholine (molar ratio)	
Cells, grown with AMG-PG (μg/ml)	Plasma membranes	Nuclei	Plasma membranes	Nuclei
MO_4 fibrosarcoma				
5 μg/ml	8.2	3.9	0.24	0.07
10 μg/ml (exp. 1)	14.6	6.4	0.51	0.13
10 μg/ml (exp. 2)	8.8	4.0	0.26	0.09
HL-60 leukemia				
0.75 μg/ml	nd[a]	nd	0.06[b]	0.02
LLC-H61 carcinoma				
30 μg/ml	16.7	7.3	0.72	0.23

[a]nd, Not determined.
[b]Measured in a crude membrane fraction (microsomes).

with the inhibitory effect of the incorporated AMG-PC on the invasiveness of the tumor cells in embryonic chick heart fragments, as shown in detail before (2). The validity of this invasion assay is well established (2,12,13); we present the results here only in a simplified semiquantitative notation (Table 3). The experimental results allow us to draw the following conclusions: First, accumulation of AMG-PC in the cellular membranes under conditions where cell growth was only minimally affected generally led to inhibition of tumor cell invasion. However, the degree of inhibition for a given cell type (MO_4) was not strictly correlated with the amount of AMG-PC incorporated. For LLC-H61 cells, the least sensitive to the cytotoxic action of AMG-PC, even massive incorporation of the agent (at 30 μg/ml) still allowed a small degree of invasion. Clearly, the inhibitory effect of AMG-PC depends on the cell type. Second, the extent of the AMG-PC–induced alterations in fluidity

TABLE 3

Summary of Data Relating Accumulation of AMG-PG from Culture Medium into Tumor Cellular Membranes with Alterations in Membrane Fluidity and Inhibition of Tumor Cell Invasiveness In Vitro

			δP_{DPH}[b]		
Cells	AMG-PC (μg/ml) in medium	Relative membrane incorporation AMG-PC[a]	Plasma membranes	Crude nuclei	Tumor cell invasiveness[c]
MO_4	5 (exp. 1)	+ +	o/+	o	+/+ +
	10 (exp. 1)	+ + +	+	o	–
	10 (exp. 2)	+ +	o	nd	–
HL-60	0.75	+	o	–	nd
LLC-H61	10	nd	o	nd	+
	30	+ + + +	+ +	–	–/+
R1C	5	nd	+ + +	o	–

[a]Deduced from Table 2.

[b]–/o/+/+ +, decrease/unaltered/relative increases in fluorescence polarization; deduced from Table 1.

[c]Semiquantitative analysis of invasion into chick heart tissue, graded according to Bracke et al. (14). –, No invasion (grades I and II); +, invasion less than 50% (grade III), + +, invasion more than 50% (grade IV). For details see Materials and Methods.

of the plasma membranes or in the cell nuclear fractions was not correlated with the degree of inhibition of invasion of the various tumor cells.

Although this study shows that no direct causal relationship exists among the amount of AMG-PC accumulated in tumor cell membranes, the consequent alterations in membrane fluidity and the extent of inhibition of tumor cell invasiveness, these membrane structural alterations nevertheless may contribute to the biological mechanisms of AMG-PC action, in which other factors may play a more dominant role (16,17).

Estimation of membrane fluidity. 1,6-Diphenyl-1,3,5-hexatriene (DPH; Koch-Light Labs, Colnbrook, United Kingdom) was used as a fluorescent probe for measuring the steady-state fluorescence polarization (P_{DPH}, at 25 C) with Elscint apparatus, model MV-1A (Elscint Ltd., Haifa, Israel) (8). P_{DPH} values mainly reflect the orientational constraint of the motions of the probe, and they are quantitatively related to order parameters in the membrane lipids (11). High P_{DPH} values represent high structural order or low membrane fluidity, and vice versa. The quantitative contributions of the individual membrane lipid components to the P_{DPH} value have been described in detail (12).

Invasion assay. The capacity of tumor cells to invade normal tissue was assayed in confrontations between test cell aggregates (diameter 0.2 mm) and precultured fragments of 9-day-old embryonic chick heart (diameter 0.4 mm) on top of a semisolid agar medium, as described in detail elsewhere (2,13). The degree of cell interaction was classified as described previously (14): grades I and II (confronting cells found at the periphery of or intermixed with the outer fibroblastic layer of the heart fragment, respectively) were combined to indicate the absence of invasion (denoted as –); grade III, when confronting cells replaced the cardiac muscle to less than 50% (denoted as +); and grade IV, when confronting cells replaced the cardiac muscle to more than 50% (denoted as + +). Grades III and IV meet the criteria of invasion in vivo.

RESULTS AND DISCUSSION

Four types of tumor cells were grown in the presence of the agent AMG-PC. The doses added to the culture media were adapted to individual cell lines according to the degree of cytotoxicity. For instance, for HL-60 cells the drug was already quite toxic at 1 μg/ml, whereas growth of LLC-H61 cells was not inhibited in the presence of a dose as high as 30 μg/ml. In all cases, the highest concentration of AMG-PC used permitted cell growth to densities of at least 75% of those observed for controls. Under these conditions, we have determined the drug-induced alterations in the structural order of membrane lipids (i.e., reciprocal of membrane fluidity; ref. 11) by steady-state fluorescence polarization using the probe DPH (Table 1). The measurements were performed in intact cells, in purified plasma membranes and in a crude nuclear fraction. No comparison should be made between the absolute P_{DPH} values of different experiments, because it is known that small differences in culture conditions (e.g., cell density, serum, time after subculturing) may already affect the data (reviewed in ref. 15). For instance, a different degree of triglyceride accumulation in cells may lead to differences in P_{DPH} values. When compared to the appropriate controls within individual experiments, the P_{DPH} values in the intact cells were in most, but not all, cases decreased in a drug dose–dependent fashion, in agreement with previous results on MO_4 cells (2). In the latter report (2), this decrease in P_{DPH} was tentatively attributed to the cellular membranes rather than triglyceride accumulation in the cytoplasm. Surprisingly, however, the present results (Table 1) demonstrate that the P_{DPH} values in the purified plasma membranes were not decreased, but rather increased in some cases (R1C; MO_4, exp. 1; LLC-H61, exp. 2),

especially at the higher doses of AMG-PC. This was why we measured other subcellular fractions, notably a nuclear one. Here we found a decrease in P_{DPH} for two cell types, HL-60 and LLC-H61. We have confirmed that this decrease indeed pertained to the structural order of the lipids of this fraction; liposomes prepared from the extracted lipids of the nuclear fractions showed a similar difference in P_{DPH} value (data not shown).

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A Continuous Assay for O-Alkylglycerol Monooxygenase (E.C. 1.14.16.5)[1]

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The antitumor activity of alkyl lysophospholipids has raised some questions concerning the degradation of O-alkyl bonds in naturally occurring ether lipids. In this report, we describe the first continuous assay for O-alkylglycerol monooxygenase (AGMO), the only enzyme known to cleave the O-alkyl bond in saturated ether lipids and ether phospholipids. AGMO activity was monitored at 340 nm by coupling the NADH redox reaction to the tetrahydropteridine cofactor of the rat liver microsomal enzyme. Turnover rates as low as 0.6 nmol/min could be measured. Using radiolabeled substrates, the products were identified with a TLC-Linear-Analyzer. The only interference with this assay can arise from other reducing agents, e.g. dithiothreitol. The assay was used to develop protocols for the solubilization of AGMO from membrane preparations in the presence of detergents.
Lipids 22, 824–830 (1987).

The antitumor activity of alkyl lysophospholipids (ALP) has been ascribed to their ability to stimulate macrophages (1) and to their cytotoxicity (2–4). One of the possible explanations for the cytotoxicity is that the tumor cells cannot degrade and therefore they accumulate (5) these lytic compounds because they lack a key enzyme (6). It is likely that the enzyme(s) cleaving O-alkyl bonds are not present in these cells (6,7). The only enzyme known to cleave the O-alkyl bond in ether lipids and ether phospholipids is O-alkylglycerol monooxygenase (AGMO, E.C. 1.14.16.5). This tetrahydropteridine (PtH_4)-dependent enzyme first was described in rat liver microsomes (8) and in tumor cells AGMO activity could not be detected or was present only at low levels (7). To facilitate more detailed investigations on the role of AGMO, we have developed a continuous spectroscopic assay.

MATERIALS AND METHODS

Enzymes and coenzymes. Dihydropteridine reductase (E.C. 1.6.99.7), *DL*-6-methyl-5,6,7,8-tetrahydropterine ($MPtH_4$) and *DL*-6,7-dimethyl-5,6,7,8-tetrahydropterine ($DMPtH_4$) were purchased from Sigma, Munich; NADH was purchased from Boehringer Mannheim, FRG.

Substrates. 1-O-[15′,16′-^{3}H]Hexadecyl-*rac*-glycerol (27 µCi/µmol, 1-[^{3}H]H-G), 3-O-hexadecyl-*sn*-glycero-1-phosphocholine (3H-*sn*-G-1PC) and 1-O-hexadecyl-*sn*-glycero-3-phosphocholine (1H-*sn*-G-3PC) were synthesized in our laboratory according to Eibl (9–11). Radiolabeled lyso-platelet-activating factor (1-O-[^{3}H]octadecylglycero-3-phosphocholine) was purchased from Amersham Buchler, Braunschweig, FRG. The tritiated substrates were dissolved in 0.1 M Tris/HCl buffer (pH 8.8) at a concentration of 10 mM. 1-[^{3}H]H-G was solubilized with 7 mg/ml tetradecylphosphocholine, which also was synthesized in our laboratory. The concentration of 3H-*sn*-G-1PC or 1H-*sn*-G-3PC was 10 mM in buffer.

Detergents. All detergents were purchased in the highest available quality from Serva, Heidelberg, FRG.

Microsomal AGMO preparation. Male rats (Wistar strain) with a body weight of 200–300 g were anesthesized with ether, decapitated, and the liver was removed. After washing with 0.9% NaCl (w/v) solution, the liver (about 7 g per animal) was cut into small pieces and homogenized with three strokes in an Elvehjem-Potter homogenizer with a teflon piston (400 rpm). For each gram of tissue, 1 ml of 0.25 M sucrose in 0.05 M Tris/HCl buffer (pH 7.6) was used. All procedures were carried out at 4 C. Microsomes were prepared by a two-step centrifugation using the method of Yawetz et al. (12). The microsomal pellet was resuspended in a 0.05 M Tris/HCl buffer (pH 8.8) that contained 20% glycerol (w/v) at a protein concentration of about 20 mg/ml.

Protein was determined according to the method of Peterson (13) with BSA as standard.

AGMO assay: spectrophotometric. A typical assay system consisted of the following compounds, which were incubated in the sample and reference cuvettes in a temperature-controlled (37 C) cell-holder of a double-beam spectrophotometer (Hitachi 150/20): 50 µl AGMO preparation (about 1 mg protein), 20 µl NADH solution (10 µmol/ml), 2.5 µg dihydropteridine reductase (5 µl) and 500 nmol alkyl lysophospholipid or 50–500 nmol alkylglycerol. Tris/HCl buffer (0.1 M, pH 8.8) was used to make up 1 ml. Sample and reference cuvettes then were adjusted at 340 nm, and optical stability was checked for 30 sec. The reaction was initiated by addition of 10 µl $MPtH_4$ (0.2 µmol) in the sample cuvette. The decrease in absorbance at 340 nm was followed for 1 min (ΔA_R). The control reaction was set up with an identical incubation mixture except lacking substrate in both cuvettes. A control without substrate in both cuvettes is necessary to detect the amount of the NADH/NAD^+-side reactions (Fig. 1, step 2) and eliminate the contribution of the autoxidation of $MPtH_4$ (step 3). The change in absorbance within one min after addition of $MPtH_4$ is ΔA_C. AGMO activity was calculated from the difference, $\Delta A = \Delta A_R - \Delta A_C$, using an absorbance coefficient $\varepsilon_{340} = 6.22\ mM^{-1}cm^{-1}$.

AGMO assay: radiolabeled substrates. The products of the spectroscopic AGMO assay with 50–100 nmol 1-[^{3}H]H-G (1.35 – 2.7 µCi per incubation) were identified

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Abbreviations: AGMO, O-alkylglycerol monooxygenase; ALP, alkyl lysophospholipids; BSA, bovine serum albumine; DTT, dithiothreitol; 1-[^{3}H]H-G, 1-O-[^{3}H]hexadecylglycerol; 3H-*sn*-G-1PC, 3-O-hexadecyl-*sn*-glycero-1-phosphocholine; 1H-*sn*-G-3PC, 1-O-hexadecyl-*sn*-glycero-3-phosphocholine; PtH_4, *D,L*-5,6,7,8-tetrahydropterine; $DMPtH_4$, *D,L*-6,7-dimethyl-PtH_4; $MPtH_4$, *D,L*-6-methyl-PtH_4; PtH_2, dihydropteridine, q-, quinoid isomer or 7,8-isomere, see Fig. 4; $DMPtH_2$, $MPtH_2$, 6,7-dimethyl- or 6-methyl-derivate, respectively, of PtH_2; TLC, thin layer chromatography.

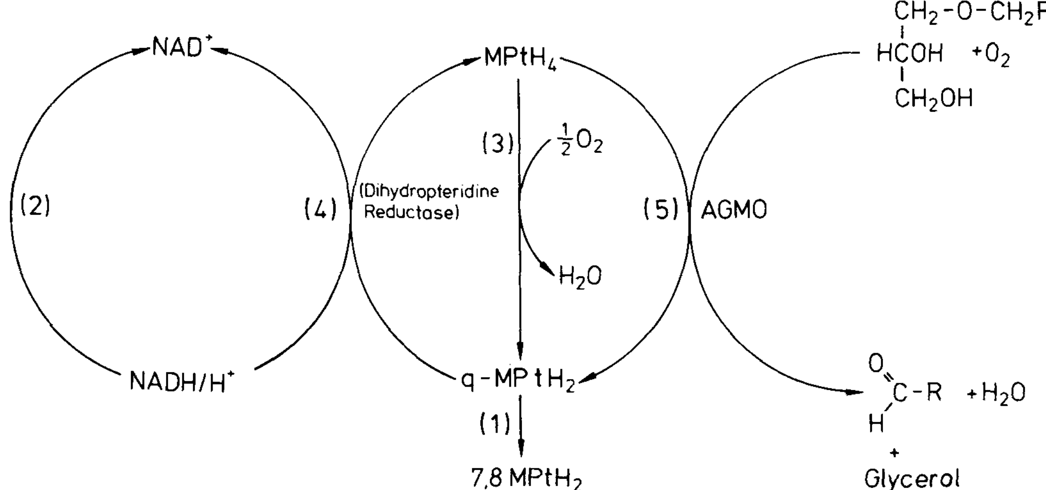


FIG. 1. Reactions in the coupled AGMO assay. The AGMO-catalyzed cleavage of the *O*-alkyl bond is shown with an alkylglycerol and oxygen as substrates and 6-methyl tetrahydropterine ($MPtH_4$) as cofactor (5). The cofactor is converted to a quinoid 6-methyl-dihydropterine (q-$MPtH_2$), which can tautomerize in a side reaction to the 7,8-isomer (1). The quinoid tautomere is regenerated to $MPtH_4$ by reductase and using NADH/H+ as cofactor (4). The resulting decrease of NADH is continuously followed at 340 nm. Side reactions and autoxidation of NADH and $MPtH_4$ are summarized under (2) and (3).

by using radiolabeled substrates. The enzyme reaction was stopped by adding 100 μl undiluted formic acid to both photometer cuvettes after the 1 min incubation. The solutions were freeze-dried in small tubes (1.5 ml), and the residue from each tube was extracted with 200 μl $CHCl_3$. Aliquots were separated on Kieselgel 60 TLC plates (Merck Darmstadt, FRG) and developed in hexane/diethylether/acetic acid (60:40:1). The radioactive products were localized and quantified on the plates with a Berthold TLC linear analyzer (Wildbad FRG; 14). Qualitative identification was performed by comparing the R_f-values with reference substances on the same TLC plate.

RESULTS

The principle of the assay is shown in Figure 1. The cleavage of the *O*-alkyl bond by the enzyme (step 5) is coupled with the oxidation of its cofactor, 6-methyl-5,6,7,8-tetrahydropterine ($MPtH_4$). We found that it is possible to use the regeneration of $MPtH_4$ by oxidation of NADH (step 4) for a continuous spectrophotometric enzyme assay. The coupled AGMO assay system described here involves several reaction steps, which we have examined in detail. The effect of the assay conditions on these reactions is described below. The data given in the tables and figures are means of three to 10 measurements with a standard deviation of ±5%.

Characterization of the products and comparison with other assay systems. Cleavage of the *O*-alkyl bond in ether phospholipids by AGMO can be demonstrated directly under the conditions used for spectrophotometric assay. As shown in Figure 2, the rate of appearance of the various radiolabeled products of oxidation of the substrate 1-[^{3}H]H-G was identified and quantified by TLC. With the mobile phase used, reaction products (hexadecanol R_f = 0.24, hexadecanoic acid R_f = 0.35, hexadecanal R_f = 0.63) were well separated from the substrate (R_f = 0.04). The appearance of products generated with time also was analyzed. After 0.5 min, hexadecanal was the main product, while hexadecanoic acid was present at the same amount with small traces of hexadecanol after one min (data not shown).

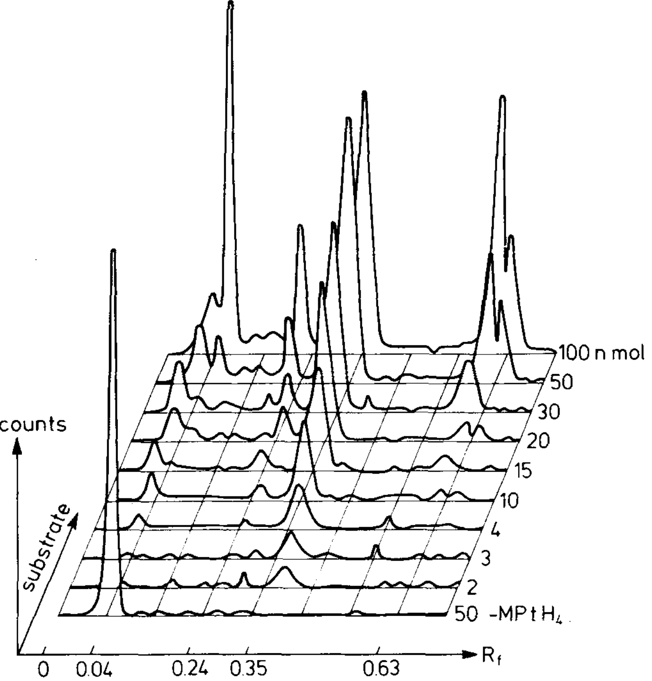


FIG. 2. Identification of products after AGMO action (1 min) with increasing concentrations of 1-[11′,12′-^{3}H]hexadecylglycerol as substrate. The products were analyzed with a linear analyzer on TLC plates and identified by their R_f values. The substrate (R_f = 0.04) was incubated with 1 mg protein of a microsomal AGMO. The products identified after one min were hexadecanol (R_f = 0.24), palmitic acid (R_f = 0.35) and hexadecanal (R_f = 0.63).

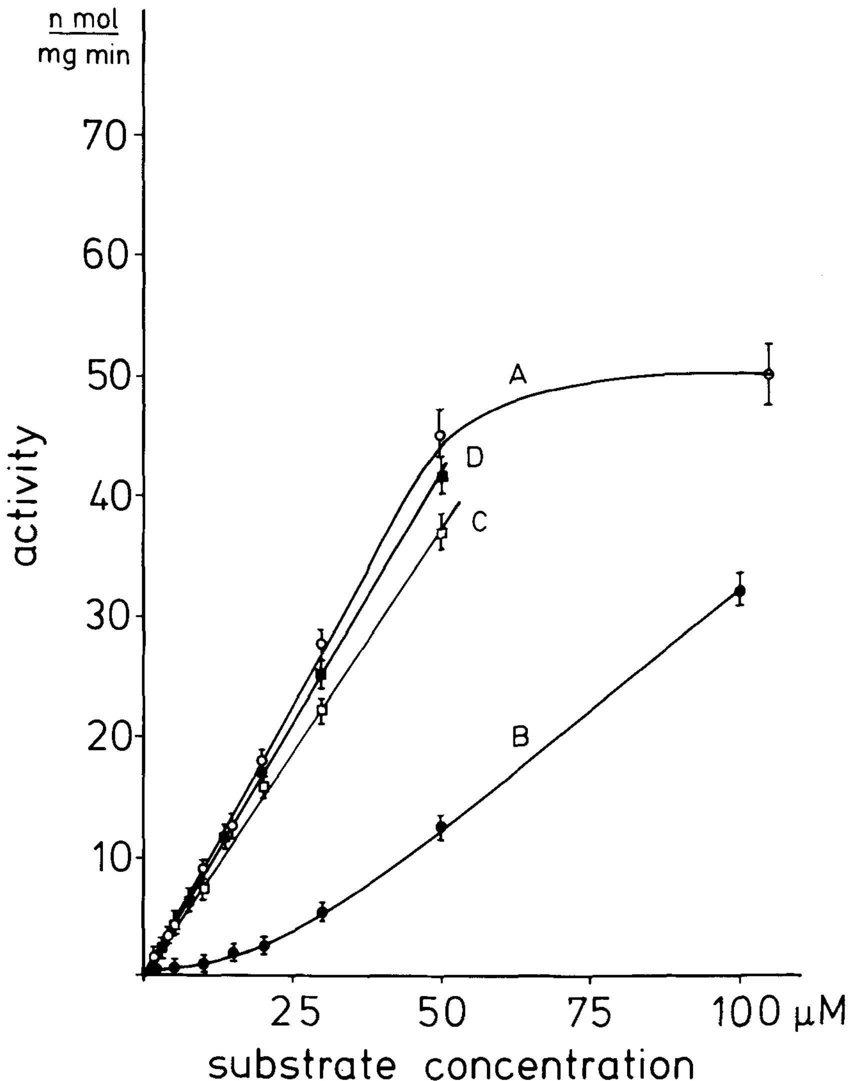


FIG. 3. True enzymatic activity within a standard deviation of 5% (curve A) is recorded only when in addition to the amount of aldehyde, generated as quantified by TLC (curve B) fatty acid and fatty alcohol are added. Curve A is measured with the spectrophotometric assay. Curve C represents the generated amounts of fatty aldehyde and fatty acid; curve D depicts the summarized amounts of fatty aldehyde, acid and fatty alcohol as calculated. As AGMO preparation, 1 mg of microsomal protein was used with 1-[11′, 12′-^{3}H]hexadecylglycerol as substrate. Incubation time for the spectrophotometric and for the radiolabeled assay was one min.

The radioactive products formed at various 1-[^{3}H]H-G concentrations during the one min incubation period are shown in Figure 2. Without the coenzyme, only the substrate is present in the incubation mixture. With increasing amounts of substrate, palmitic acid is the main product; hexadecanal (R_f = 0.63) is an intermediate that is spontaneously oxidized to hexadecanoic acid. Under the assay conditions, the aldehyde also can be reduced to hexadecanol by NADH.

Evaluation of each single product formed from 1-[^{3}H]H-G as substrate and comparison with the spectrophotometric assay are shown in Figure 3. Curve A shows the activity measured by absorbance change. In curve B, the amount of generated fatty aldehyde is shown. However, true AGMO activity data can be performed through product analysis. This was accomplished by adding the amounts of generated acid (calculated addition to the aldehyde: curve C), generated alcohol and aldehyde (curve D).

In order to compare the present continuous AGMO assay with the phosphate assay (15) and the assay conditions used by Soodsma et al. (16), microsomal enzymic activity was measured in parallel. With 1-*O*-[^{3}H]octadecyl-G-3PC as substrate, the following AGMO activities (nmol/mg min) were obtained: 7.4 ± 0.4 (this publication); 7.04 (16) and 7.1 (15). Regardless of the detection method, the AGMO activities of a microsomal enzyme preparation are the same within expected tolerance.

Choice of the cofactor. The PtH_4 cofactor is oxidized to PtH_2, and PtH_4 rapidly is regenerated by reductase using NADH/H^+ as cofactor (Fig. 1, step 4). The PtH_4 cofactor is very sensitive to autoxidation (Fig. 1, step 3); the product (PtH_2) occurs in two isomeric forms, quinioid-

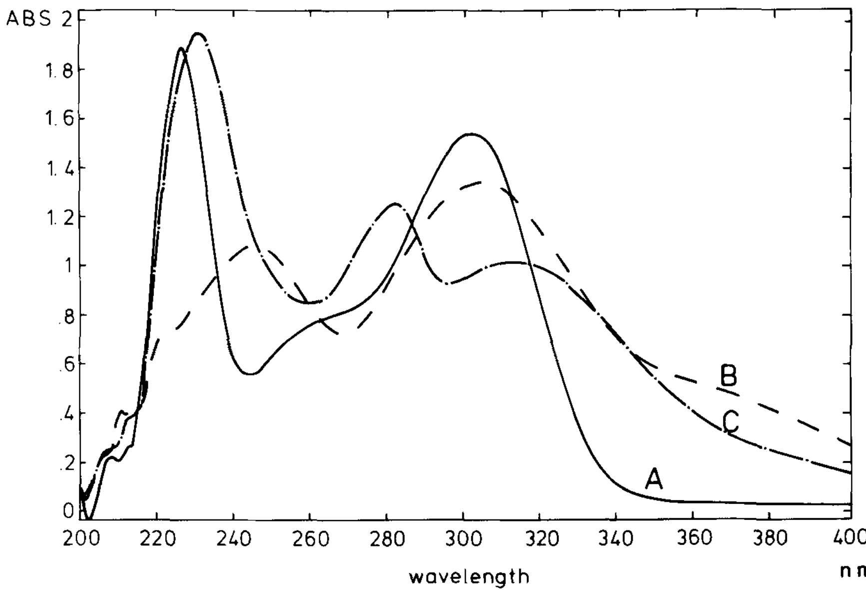


FIG. 4. UV-vis spectra of *D,L*-6-methyl-5,6,7,8-tetra-hydropterine ($MPtH_4$) (A), of quinoide *D,L*-6-methyl-dihydropterine (B) and of 7,8-*D,L*-methyl-dihydropterine (C). The spectra were recorded with a Hewlett-Packard 8451A diode array spectrophotometer. A total volume of 1 ml 0.1 M Tris/HCl buffer (pH 8.8) contained 0.2 μmol $MPtH_4$ (A). Curve B was recorded immediately after addition of 10 μg peroxidase (E.C. 1.11.1.7, Boehringer Mannheim, FRG) and 0.2 μmol H_2O_2. Spectrum C was recorded after 10 min.

TABLE 1

Relationship between AGMO[a] Activity and Tetrahydropteridine Structure Measured with the Spectrophotometric (A) and Radiolabeled (B) Assay

		Specific activity (nmol/mg min)			
	Assay:	A		B	
Substrate	Coenzyme:	$MPtH_4$	$DMPtH_4$	$MPtH_4$	$DMPtH_4$
3-*O*-Hexadecyl-*sn*-glycero-1-PC		4.9	1.3	—	—
1-*O*-[11′,12′-³H]Hexadecylglycerol		13.5	3.1	12.9	12.9

[a]A microsomal AGMO preparation was used, and 1 mg protein was incubated for each test. Substrate concentration was 0.5 mM for 3-*O*-hexadecyl-*sn*-glycero-1-PC and 50 μM for 1-*O*-[11′,12′-³H]hexadecylglycerol.

(q-) and 7,8-PtH_2. At 340 nm, the absorbance spectra of q-$MPtH_2$ and 7,8-$MPtH_2$ show an isobestic point (Fig. 4). Due to the higher instability of $DMPtH_4$, comparable spectra of this cofactor were not recorded.

To study the influence of structural variations in tetrahydropteridines on their cofactor properties in the continuous assay, $MPtH_4$ and $DMPtH_4$ were used in both the photometric and the radiolabeled test. 3H-*sn*-G-1PC and 1-[³H]H-G served as substrates. The results summarized in Table 1 show there is no difference in AGMO activity with both of these cofactors in the radiolabeled assay. With the spectrophotometric test, the true enzymic activity is measured only when $MPtH_4$ is used as cofactor but not with $DMPtH_4$.

Effect of reducing agents and dihydropteridine reductase. Reducing agents, such as dithiothreitol (DTT), interfere with the spectrophotometric assay because of their competition with the NADH-dependent reduction of q-$MPtH_2$ (Fig. 1, step 4). For example, addition of 1 mM DTT decreases the kinetically measurable AGMO activity to 58%, while there is no change in the detectable substrate turnover using the radiolabeled assay.

Presence of the dihydropteridine reductase (E.C. 1.6.99.7) has no significant effect on AGMO activity measured with the kinetic or radiolabeled assay because q-$MPtH_2$ is reduced spontaneously by NADH. We routinely add dihydropteridine reductase, although it is not necessary for these experiments.

Effect of detergents. To show the effect of detergents towards AGMO activity, rat liver microsomes were incubated with various concentrations of detergents as described in Table 2. AGMO is hardly affected by detergents such as digitonin and is inactivated by about 50% by Triton X-100 (Table 2). Centrifugation of the

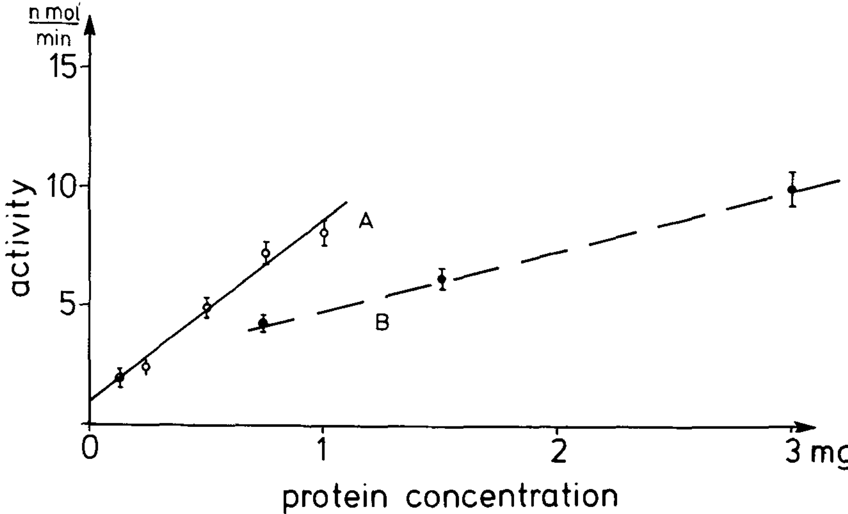


FIG. 5. Relationship between substrate cleavage and protein concentration of the AGMO preparation for a microsomal (A) and a digitonin-solubilized sample (B). As substrate, 3-*O*-hexadecyl-*sn*-glycero-1-phosphocholine (0.5 mM) was used. AGMO activity was estimated with the new continuous assay and values were calculated from the initial rate of one min incubations.

TABLE 2

Effect of Different Detergents on AGMO Activity

Detergent	Conc. (%, w/v)	AGMO activity (%) Incubation	AGMO activity (%) Supernatant
None	—	100	3
Octylglucoside	0.8	54	10
Triton X-100	0.08	50	4
	0.16	46	17
Digitonin	0.50	100	0
	1.5	98	76
Nacholate	0.3	66	0
Nadesoxycholate	0.05	63	7

Rat liver microsomes (AGMO activity 7.4 nmol/mg min = 100%, substrate: 3H-*sn*-G1PC) were incubated with the given detergent concentrations in 0.05 M Tris/HCl buffer (pH 8.8) on ice for 15 min. AGMO activity was measured again; the mixture then was centrifuged for one hr at 200,000 × g in a Beckman ultracentrifuge. AGMO activity was estimated in the supernatant as described in Materials and Methods.

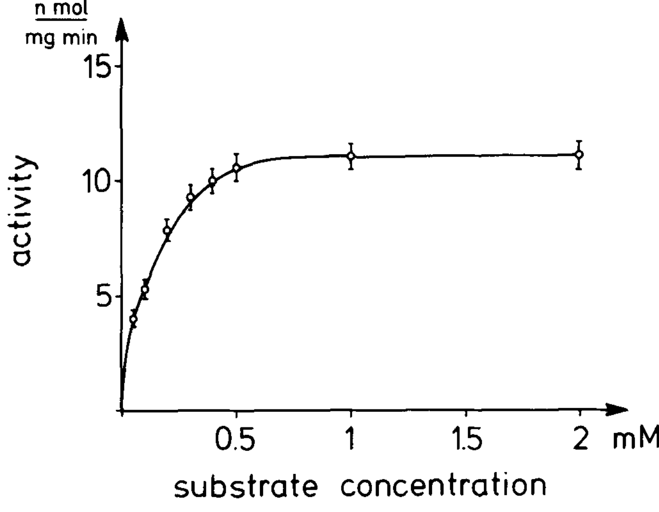
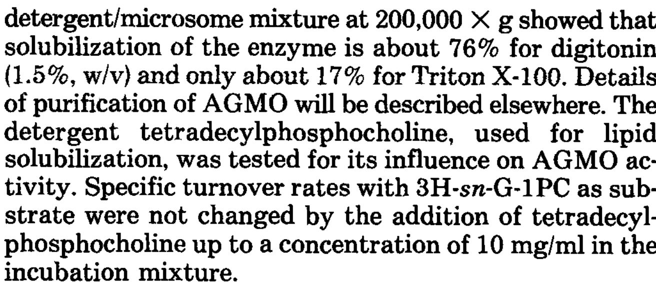


FIG. 6. Relationship between AGMO activity and 3-*O*-hexadecyl-*sn*-glycero-1-phosphocholine concentration. A microsomal AGMO preparation (1 mg) was used in the spectrophotometric assay; enzymic activity was calculated from the initial rate of one min incubations.


detergent/microsome mixture at 200,000 × g showed that solubilization of the enzyme is about 76% for digitonin (1.5%, w/v) and only about 17% for Triton X-100. Details of purification of AGMO will be described elsewhere. The detergent tetradecylphosphocholine, used for lipid solubilization, was tested for its influence on AGMO activity. Specific turnover rates with 3H-*sn*-G-1PC as substrate were not changed by the addition of tetradecylphosphocholine up to a concentration of 10 mg/ml in the incubation mixture.

Linearity with protein concentration. The initial rate of cleavage of the alkyl bond is linear with protein concentration (Fig. 5) for the microsomal AGMO preparation (curve A). Higher (>1 mg/ml) concentration of microsomal preparations could not be measured because of light-scattering effects. With AGMO solubilized in digitonin (1.5%, w/v), the linearity of the activity could be shown in the range of 0.75 mg to 3 mg protein. The lowest amount of AGMO activity that could be detected was 0.6 nmol/min, corresponding to 34 μg protein of rat liver microsomes in 1 ml.

Dependence on the substrate concentration and the substrate specificity of AGMO. Dependence of the reaction rate on the substrate concentration is shown in Figure 6. The initial rate of AGMO activity shows saturation at a concentration of 0.5 mM 3H-*sn*-G-1PC. The curved shape and the saturation concentration agree with

TABLE 3

Substrate Specificity of AGMO[a] Determined with the Spectrophotometric Assay

Substrate	Specific activity (nmol/mg min)
3-*O*-Hexadecyl-*sn*-glycero-1-PC	4.9
1-*O*-Hexadecyl-*sn*-glycero-3-PC	4.1
1-*O*-[^{3}H]Octadecylglycero-3-PC	3.2
1-*O*-[11′,12′-^{3}H]Hexadecylglycerol	9.4

[a]A microsomal AGMO preparation was used; 1 mg protein was incubated for each test. Substrate concentraton was 0.5 mM for alkyl lysophospholipids and 50 μM for 1-*O*-[11′,12′-3H]hexadecylglycerol.

results of earlier studies (15,17). The saturation concentration for alkylglycerols is between 0.05 and 0.5 mM, depending on chain length.

The specific activity of AGMO for several substrate analogs is summarized in Table 3. 3H-*sn*-G-1PC (100%) is a slightly better substrate than the naturally configurated 1H-*sn*-G-3PC (83%). Also, hexadecyl ether lipids are better substrates than octadecyl ethers. In general, long chain alkylglycerols are better substrates than ALP (Table 3, see also ref. 18).

DISCUSSION

The present assay offers some obvious advantages over those proposed earlier (15,16,19,20). The specific activity in the microsomal fraction and the dependence on the substrate concentration are in accord with those observed by others. The advantages of the new AGMO assay developed here are:

- It is fast, simple and sensitive.
- The assay is continuous, and initial rates can be measured by using reasonably small samples of the enzyme and the substrate.
- There are no structural restrictions for substrate specificity studies like a radiolabel or phosphate group in the molecule.
- No substrate/product separations are necessary.
- The high sensitivity of the assay enables, after a few preparation steps, detection of AGMO in different cell and tissue samples.

The variability of products formed during enzymatic hydrolysis observed by other authors (8,16,17,21,22) could be due to autoxidation of the primary product. The cleavage products in the presence of nucleotides has been established before (16). In accordance, our TLC analysis shows that fatty alcohol, aldehyde and acid were formed. Under the incubation conditions of the spectroscopic assay, NADH is a strong reducing agent for PtH_2. Therefore, PtH_2 produced during *O*-alkyl cleavage immediately generates NAD^+, which may spontaneously oxidize the aldehyde to acid. The suggestion of Pfleger et al. (21) that fatty aldehyde is an intermediate product whose fate in the incubation medium is dependent on other agents present is valid.

As shown in Table 1, it is more advantageous to use $MPtH_4$ than $DMPtH_4$ as a cofactor for AGMO in the continuous assay. This is because the rate of isomerization (Fig. 1, step 1) of $DMPtH_2$ is sensitive to the buffering ions (23), and the tautomerization of q-$DMPtH_2$ is twice as fast as that of q-$MPtH_2$ (23). In addition, q-$MPtH_2$ is reduced quantitatively by NADH to $MPtH_4$, while q-$MPtH_2$ is reduced only 25% to $DMPtH_4$ (Table 1). This explains the observation of Tietz et al. (8) that $MPtH_4$ has significantly better cofactor properties than $DMPtH_4$ in the presence of reduced nucleotides. Van der Heiden and Brink (24) raised some objections against the use of NADH detection at 340 nm in the presence of $MPtH_4$ because they observed a shift of the absorbance maximum of NADH to a longer wavelength. Also, they described that the nonenzymatic oxidation of NADH with q-$MPtH_2$ is not linear with time. Under the AGMO assay conditions described here (using the initial phase of the enzymic reaction), these difficulties were resolved and measurement of the initial rate of the enzyme reaction was possible.

The present assay was employed to monitor the purification of AGMO. As shown in Table 2, the use of digitonin to solubilize AGMO from microsomal membranes is more effective than Triton X-100, as described by Ishibashi and Imai (22). When comparing specific activity with 1-[^{3}H]H-G as substrate, one must consider that activities used by the authors are given for an incubation time of 30 min. Compared to specific activity of 0.54 nmol/mg min (normalized from the 30 min time points) as reported by these authors, our solubilized microsomal AGMO preparations have an average specific activity of 10.8 nmol/mg min. We will report the purification of the enzyme in detail elsewhere.

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Substrate Specificity of *O*-Alkylglycerol Monooxygenase (E.C. 1.14.16.5), Solubilized from Rat Liver Microsomes[1]

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Synthetic alkyl lysophospholipids (ALP) show antineoplastic activity. In the present discussion, the cytotoxicity of ALP is attributed to their accumulation in tumor cells. Some neoplastic cell species, in contrast to normal cells, cannot metabolize ALP because of a lack of *O*-alkylglycerol monooxygenase (AGMO) activity. To understand the metabolic fate of ether lipids and ether-linked phospholipids, AGMO substrate specificity studies were undertaken. Thirty-five different natural and synthetic ether lipids and their metabolites (including a thioether) were tested as substrates for AGMO. The study revealed that the potent cytostatic substance, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine is not a substrate for AGMO. Therefore, its selective toxicity to tumor cells cannot be based on the differences in direct detoxification of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine by AGMO in normal and malignant cells. However, 1-*O*-octadeyl-2-*O*-methyl-*rac*-glycerol, which can be formed by phospholipase C hydrolysis, is a good substrate for AGMO.
Lipids 22, 831–835 (1987).

The antitumor activity of synthetic alkyl lysophospholipids and lysophospholipid analogs (ALP) has been ascribed to a direct cytotoxic effect (1–4). This direct effect and the cause of ALP selectivity against tumor cells still is not clearly understood (5,6). As originally shown by Snyder and Wood (7,8), various tumors of animal and human origin have high levels of ether lipids. It has been proposed that this enrichment of ether lipids in some tumor cell species is due to the lack of ether lipid-degrading enzyme activity (9). Such an enzyme is alkylglycerol monooxygenase (AGMO), which originally was discovered in rat liver microsomes (10). Indeed, it has been shown that many tumor tissues are low in AGMO levels (11). Therefore, ALP could accumulate in tumor cells, leading to disturbance of lipid metabolism (12). AGMO is the only known enzyme to cleave the *O*-alkylbond in saturated ether lipids according to the reaction scheme below (10), using tetrahydropteridine (PtH_4) derivatives as a cofactor. Fatty aldehyde and glycerol or glycerophosphocholine are the primary cleavage products in this reaction (Fig. 1).

Although several investigations on AGMO have been carried out in the past (3,10,13–17), there still is lacking clear-cut information on the substrate specificity of an enriched AGMO preparation from rat liver. To obtain more information on the metabolism of natural (e.g. PAF) and synthetic ALP, we have undertaken a detailed study of the general structural requirements of AGMO substrates. For this, we developed a new assay for AGMO that uses the $NADH/NAD^+$ redox reaction to measure continuously the decrease of the tetrahydropteridine cofactor. A detailed report on this assay is published in this issue (18). With this continuous assay, we were able to measure the substrate properties of a large variety of ether lipids that were synthesized in our laboratory without the need for a radioactive label or for a phosphate group in the molecule; these are prerequisites for the use of other assay methods for AGMO activity (14,17). In addition, the kinetic analysis of *O*-alkyl cleavage reaction is performed easily with this spectrophotometric assay. AGMO substrate specificity was measured with a microsomal enzyme preparation and the digitonin–solubilized enzyme from rat liver (18).

EXPERIMENTAL

Ether lipids. All lipids were synthesized in our laboratory according to Eibl (19–21). The substrates were dissolved in 0.1 M Tris/HCl buffer (pH 8.8) in the concentrations given in the tables. Compounds that were difficult to dissolve in buffer were solubilized with 1-lauroyl-propanediol-1,3-PC (7 mg/ml) or tetradecyl-PC (7 mg/ml)

```
  H2C-O-CH2-R     PtH4        PtH2   H2C - OH
    |                                   |
HO-C-H        +O2  ----------------> HO - C - H   +RCHO+H2O
    |                                   |
  H2C-O-X               AGMO         H2C - O - X
  (X = PC , H)
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FIG. 1.

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Abbreviations: AGMO, *O*-alkylglycerol monooxygenase; ALP, alkyl lysophospholipid; PAF, platelet-activating factor; PC, phosphocholine; PtH_4, *D,L*-6-methyl-5,6,7,8-tetrahydropterine, PtH_2, *D,L*-6-methyl-dihydropterine, TLC thin layer chromatography.

containing buffer. Lysoplasmalogens were isolated according to the procedures of Warner and Lands (22) and Pugh et al. (23) from bovine heart; they were purified by silica gel chromatography and characterized by TLC.

Other chemicals. All other chemicals purchased were the highest available quality and used without further purification.

Preparation of O-alkylglycerol monooxygenase. Rat liver microsome preparation and solubilization of AGMO with the use of digitonin has been described before (18).

Enzymatic assay. Turnover rates and specific AGMO activities were estimated with the coupled spectrophotometric assay at 37 C in 0.1 M Tris/HCl buffer (pH 8.8) with the cofactor *D,L*-6-methyl-5,6,7,8-tetrahydropterine (0.5 mM) as described elsewhere (18). In each assay, 0.8 mg protein of a microsomal AGMO preparation or 1.5 mg protein of a digitonin (1.5%, w/v) solubilized AGMO sample was added to the reaction mixtures in a total volume of 1 ml. By variation, optimal substrate concentrations were estimated for each compound and given in the tables. Absorbance change was read on a Hitachi 150-20 double-beam spectrophotometer for two min, and AGMO activity was calculated from the initial rate.

Protein estimation. Protein concentration was determined according to the method of Peterson (24) with bovine serum albumin as standard.

TABLE 1

O-Alkylglycerol Monooxygenase (AGMO) Activity Solubilized by Digitonin from Rat Liver Microsomes with Mono-*O*-alkylglycerols and 1-*S*-Octadecylglycerol as Substrates

Substrate (-*rac*-glycerol)	Conc.[c] (mM)	AGMO activity (nmol/min/mg)	(%)[d]
1-*O*-propyl-	0.5–90	0	0
1-*O*-pentyl-	1–90	0	0
1-*O*-hexyl-	50	6.2	94
1-*O*-heptyl-	15	3.6	54
1-*O*-octyl-	10	3.6	55
1-*O*-nonyl-	1	0.4	5.3
1-*O*-dodecyl-[a]	2	3.5	53
1-*O*-tetradecyl-[a]	1	5.1	78
1-*O*-hexadecyl-[b]	0.5	12.7	192
1-*O*-octadecyl-[b]	0.5	7.4	112
1-*S*-octadecyl-[b]	1	5.5	83
1-*O*-octyl-*D*-mannitol	0.5–10	0.3	4

[a]Substrate was solubilized with 1-lauroyl-propanediol-1,3-PC (7 mg/ml).
[b]Substrate was solubilized with tetradecyl-PC (7 mg/ml).
[c]Optimal substrate concentration for the highest AGMO activity is given.
[d]3-*O*-Hexadecyl-*sn*-glycero-3-phoscholine is set to 100%.

RESULTS

The specificity of AGMO for various substrates was measured with membrane-bound as well as with digitonin-solubilized AGMO enzymes. Specific AGMO activity with 3-*O*-hexadecyl-*sn*-glycero-1-PC was 4.9 nmol/mg/min for microsomes and 6.6 nmol/mg/min for the digitonin extract, and the values were defined as 100% activity. This compound was chosen as a standard because it is easily water-soluble, forming micelles without addition of detergents. Also, it was the best ether phospholipid substrate for AGMO. The data given in the Tables 1–4 are means of three to 10 measurements with a standard deviation ±5%.

The substrate behavior of 1-*O*-alkylglycerols with the digitonin–solubilized enzyme is summarized in Table 1. In view of their substrate properties, *O*-alkylglycerols can be divided into three groups: first, the monomeric soluble short chain alkylglycerols (propyl- and pentyl-residues), which are not cleaved by AGMO; and second, the ether compounds with a C_6 to C_9 alkyl residue. These hexyl- to nonyl-*O*-glycerols are substrates for AGMO with activities depending on their maximal nonaggregated concentrations. The third group, the long chain *O*-alkylglycerols (C_{14} to C_{18}), are very good substrates; the hexadecyl residue is the optimal chain length (192%). The large differences in the optimal substrate concentrations for AGMO activity resulted from the different critical micellar concentrations of each compound (short-chain and long-chain alkyl ethers).

Lauroylpropanediol-1,3-PC and tetradecyl-PC, used as detergents for lipid solubilization, were tested for their influence on AGMO activity. Specific turnover rates of 3-*O*-hexadecyl-*sn*-glycero-3-PC were not changed by addition of these lipids to an incubation mixture of up to 10 mg/ml of each compound.

Thioethers, for instance, 1-*S*-octadecylglycerol, also are cleaved by AGMO. The rate is lower than for the corresponding ethers.

Investigations on the influence of the monomeric solubility of the ether glycerides on their substrate properties towards AGMO were conducted using ether lipids with *D*-mannitol instead of glycerol. For instance, 1-*O*-octyl-*D*-mannitol is easily water–soluble, and this variance in the structure of the polyhydroxy backbone leads to a dramatic loss of AGMO activity (only 4%) compared to that of the 1-*O*-octylglycerol.

The substrate properties of di-*O*-alkylglycerols are summarized in Table 2. 1-*O*-Hexadecyl-3-*O*-methyl-*rac*-glycerol is the best AGMO substrate tested. However, with increasing length of the second alkyl chain, AGMO activity diminishes. As compared to the standard, the rate of hydrolysis for 1-*O*-hexadecyl-3-*O*-octyl-*rac*-glycerol is only 17%, whereas 1-*O*-tetradecyl-2-*O*-octadecyl-*rac*-glycerol does not serve as a substrate at all.

To test whether the high turnover rates of the short chain 1-*O*-hexadecyl-3-*O*-alkyl-*rac*-glycerols are due to an additive cleavage of the second ether bond, we synthesized different *O*-alkyl-palmitoyl-glycerols with alkyl chains from C_2 to C_5. None of these compounds, for instance 1-palmitoyl-3-*O*-allyl-*rac*-glycerol, is an AGMO substrate, indicating that alkyl residues shorter than C_5 are not cleaved by AGMO. Therefore, it is tempting to speculate that there is no additive cleavage of the second *O*-alkyl bond in short-chain 1-*O*-hexadecyl-3-*O*-alkyl-*rac*-glycerols.

The presence of an ester bond at the glycerol vicinal to the ether bond is a negative factor for AGMO activity,

TABLE 2

O-Alkylglycerol Monooxygenase (AGMO) Activity Solubilized by Digitonin from Rat Liver Microsomes with Di-O-alkylglycerols, Acylalkylglycerols and Derivatives as Substrates

Substrate (*rac*-glycerol)	Conc.[c] (mM)	AGMO activity (nmol/min/mg)	AGMO activity (%)[d]
1-*O*-hexadecyl-3-*O*-methyl-[b]	0.5	15	228
1-*O*-allyl-3-*O*-hexadecyl-[b]	0.5	14.1	214
1-*O*-hexadecyl-3-*O*-pentyl-[b]	0.5	7.3	110
1-*O*-hexadecyl-3-*O*-octyl-[b]	0.5	1.1	17
1-palmitoyl-3-*O*-allyl[b]	0.1–10	0	0
1-*O*-hexadecyl-2-acetyl-[a]	1	5	75
1-*O*-allyl-2-*O*-octadecyl-[a]	1	6	91
1-*O*-tetradecyl-2-*O*-octadecyl-[a]	0.1–10	0	0
1-*O*-octadecyl-2-*O*-methyl-[a]	1	6.6	100

[a]Substrate was solubilized with 1-lauroyl-propanediol-1,3-PC (7 mg/ml).
[b]Substrate was solubilized with tetradecyl-PC (7 mg/ml).
[c]Optimal substrate concentration for the highest AGMO activity is given.
[d]3-*O*-Hexadecyl-*sn*-glycero-3-phosphocholine is set to 100%.

TABLE 3

O-Alkylglycerol Monooxygenase (AGMO) Activity Solubilized by Digitonin from Rat Liver Microsomes with Alkyl-(lyso-)phospholipids as Substrates

Substrate (phosphocholine)	Conc.[a] (mM)	AGMO activity (nmol/min/mg)	AGMO activity (%)
1-*O*-hexadecyl-*sn*-glycero-3-	0.5	5.5	83
3-*O*-hexadecyl-*sn*-glycero-1-	0.5	6.6	100
1-*O*-octadecyl-*sn*-glycero-3-	0.5	4.3	65
1-*O*-hexadecyl-propanediol (1,3)-	0.5	5.7	86
1-*O*-hexadecyl-propanediol(1,2)-	0.5	3.6	54
1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-	1	1.5	22
1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-	0.1–2	0	0
1-*O*-hexadecyl-*sn*-glycero-phospho-*N,N,N*-trimethyl-hexanolamin	0.5	3.7	56

[a]Optimal substrate concentration for the highest AGMO activity is given.

as shown with 1-*O*-hexadecyl-2-acetyl-*rac*-glycerol. In comparison to 1-*O*-allyl-2-*O*-octadecyl-glycerol (91%) or 1-*O*-octadecyl-2-*O*-methyl-glycerol (100%), this compound is still a sufficient substrate for AGMO (75%).

The distance between alkyl chains on the glycerol molecule (1,2 vs 1,3, e.g. 1-*O*-allyl-3-*O*-hexadecyl-*rac*-glycerol 214%, compared with 1-*O*-allyl-2-*O*-octadecyl-*rac*-glycerol 91%) significantly influences AGMO activity. 1-*O*-Allyl-2-*O*-octadecylglycerol still is a good substrate (91%) even when one considers that in general, cleavage of a C_{18} residue occurs 1.7 times slower than the cleavage of a C_{16} chain (1-*O*-hexadecylglycerol compared to 1-*O*-octadedcylglycerol in Table 1).

Some of the ether phospholipids whose properties as AGMO substrates were investigated are listed in Table 3. Comparison of the activities of AGMO on 1-*O*-hexadecyl-*sn*-glycero-3-PC and 3-*O*-hexadecyl-*sn*-glycero-1-PC revealed that the structure with PC in the *sn*-1 position is a slightly better substrate. This is surprising, since naturally occurring phospholipids normally have the opposite configuration. AGMO again cleaves the octadecyl chain at lower rates than the hexadecyl chain, for instance, with activities of 65% for 1-*O*-octadecyl-*sn*-glycero-3-PC vs 83% for 1-*O*-hexadecyl-*sn*-glycero-3-PC. Absence of the hydroxygroup in the ALP (1-*O*-hexadecyl-propanediol-1,3-PC, 86%) does not change the substrate behavior significantly. In contrast, the close vicinity of PC to the alkyl ether bond (1-*O*-hexadecyl-propanediol-1,2-PC, 54%) has a negative influence on the substrate property.

Although the biologically important compound PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-PC) is a poor AGMO substrate, it still can be degraded. However, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-PC cannot.

Structural variations in the PC residue of ALP influence AGMO activity markedly: it decreases from 83% for 1-*O*-hexadecyl-*sn*-glycero-3-PC to 56% for 1-*O*-hexadecyl-*sn*-glycero-3-phospho-*N,N,N*-trimethylhexanolamine.

Unsaturated alkyl ether groups behave in different ways as substrates for AGMO, depending on the position of the double bond in the alkyl chain as shown in Table 4. Substitution of octadecyl by octadecen-(9,10)-yl has no significant effect on AGMO activity, as can be seen from the comparison of 1-*O*-oleyl-*rac*-glycerol and 1-*O*-oleyl-2-*O*-methyl-*rac*-glycerol (Table 4) with the octadecyl compounds 1-*O*-octadecyl-*rac*-glycerol (Table 1) and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol (Table 2), respectively. However, lysoplasmalogen, with unsaturation in the α,β-position, is not a substrate for AGMO. This is consistent with the proposed mechanism of the cleavage of the *O*-alkylbond via a hemiacetal by Tietz et al. (10). However, a slight shift of the double bond to the β-position will result in substrate properties. 1-*O*-(*cis*)-2′-hexadecenylglycerol has a rate of 124%, which is lower than the 192% that is determined for 1-*O*-hexadecylglycerol. A hemiacetal cannot be formed when the double

TABLE 4

O-Alkylglycerol Monooxygenase (AGMO) Activity Solubilized by Digitonin from Rat Liver Microsomes with Substrates Containing Unsaturated Alkyl Chains

Substrate	Conc.[c] (mM)	AGMO activity (nmol/min/mg)	(%)[d]
1-*O*-oleyl-*rac*-glycerol[a]	1	7.7	116
1-*O*-oleyl-2-*O*-methyl-*rac*-glycerol[a]	1	6.6	100
1-*O*-oleyl-2-acetyl-*rac*-glycerol[a]	1	8.8	134
lysoplasmalogen	2–200	0	0
1-*O*-(*cis*)-2′-hexadecenyl-glycerol[b]	0.5	8.2	124

[a]Substrate was solubilized with 1-lauroyl-propanediol-1,3-PC (7 mg/ml).

[b]Substrate was solubilized with tetradecyl-PC (7 mg/ml).

[c]Optimal substrate concentration for the highest AGMO activity is given.

[d]3-*O*-Hexadecyl-*sn*-glycero-3-phosphocholine is set to 100%.

bond is in α,β position to the *O*-alkylbond. Therefore, the metabolism of the *O*-alkylbond in plasmalogens by plasmalogenases and the cleavage of the saturated ether bond are two totally separate pathways.

DISCUSSION

Since phospholipids form aggregates in aqueous solutions (2), the enzymatic activities of lipid-metabolizing enzymes could be related to the lipid superstructures and surface properties (25,26). A study of AGMO activity toward short- and middle-chain alkyl glycerols showed that these compounds are substrates only in a very small range of concentration. So, it is not surprising that e.g. 1-*O*-octylglycerol was not described as a substrate for AGMO in a previous study (10). As is shown in detail elsewhere (27), AGMO is a membrane-bound enzyme. This fact makes the development of model kinetics of the substrate behavior difficult, and normal kinetic analyses are not valid (28). However, microsomal fractions show the same AGMO specificity as solubilized and enriched enzyme samples do (data not shown). In addition, with 1-*O*-hexadecyl-*rac*-glycero-3-PC and 1-*O*-hexadecylglycerol as substrates, the saturation of substrate concentration are in accord with data from other investigations (15,17).

The poor solubility of the long-chain *O*-alkylglycerols (C_{12} to C_{18}) in water raises several difficulties in the assay system, such as low substrate concentration and turbidity. Several methods for overcoming these problems have been used by others (10,13–16,29–31) based on using alcohols to dissolve the substrates. As one can observe in a cuvette, addition of an alcoholic solution or of substrate to the aqueous assay system immediately leads to precipitation of the substrate. A powerful help is using the phospholipid analogs such as 1-lauroyl-propanediol-1,3-PC and tetradecyl-PC as detergents, resulting in a clear solution of the substrates. Within the concentration range used, these compounds had no effect on AGMO activity. Using these detergents is an additional reason why our microsomal AGMO activities with substrates like 1-*O*-hexadecylglycerol are higher than those reported by others (10,14–16).

While PAF shows only poor substrate property (22%, Table 3), the lyso-compound 1-*O*-hexadecyl-*sn*-glycero-3-PC (Lyso-PAF) is a good substrate (83%, Table 3). Lyso-PAF is generated by acetyl hydrolase (E.C. 3.1.1.48) (32) removing the acetyl residue from the *sn*-2 position. This is the biological-important pathway in PAF metabolism. In addition, phospholipase C (E.C. 3.1.4.3) acts on PAF with very high turnover rates (33). The product, 1-*O*-hexadecyl-2-acetyl-glycerol, is an intermediate in PAF biosynthesis (34) and also is a good substrate for AGMO (75%, Table 2). Therefore, in contrast to Lee et al. (31) and in accord with Hoffman et al. (35), the hydrolysis of *sn*-2 position is not a necessary condition for the action of AGMO. In addition, the initial rate of cleavage was linear with time. This does not indicate any substrate conversion other than alkyl cleavage activity.

Starting from 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-PC, described as a potent cytotoxic agent (1), an analog cascade of enzymatic reactions can be constructed. With two different assay systems (17,18,27), it is now shown in disagreement to earlier discussion (3,5) that 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-PC is not a substrate for AGMO (Table 3). However, after removing PC through phospholipase C or a phosphocholine transferase (6), 1-*O*-octadecyl-2-*O*-methylglycerol could be generated. This compound is, like Lyso-PAF, an excellent substrate (100%, Table 2) for AGMO. To what extent ALP(-analogs) antitumor drugs can be generated selectively through competition of metabolizing enzymes is under study in our laboratory. Therefore, the simple explanation for the toxicity and selectivity of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-PC, based on the difference of AGMO content in normal and malignant cells, is not correct. This also was shown in other studies (35,36) comparing AGMO activity levels in different cell types with the sensitivity of these cells to an ALP treatment.

To recapitulate, general conclusions seem to emerge about the substrate specificity from the studies reported in this paper. The position of the ether bond to be cleaved in the glycerol backbone is not important. One steric unhindered position in the glycerol (-derivative) must be present. The substrate requires an aliphatic chain longer than C_6. In di-*O*-alkylglycerols, only one chain should be longer than C_5. In mixed acylalkylglycerols, the alkyl chain to be cleaved must be longer than the acyl residue. The alkyl chain may contain double bonds but not in α,β position relative to the ether bond. The rate of the *O*-alkyl cleavage is dependent on structural variations in the polar region of ether lysophospholipids. Thioether lipids are also cleaved by AGMO.

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Differential Effects of Ether Lipids on the Activity and Secretion of Interleukin-1 and Interleukin-2[1]

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Alkyl lysophospholipids (ALP) are synthetic analogues of lysophosphatidylcholine and represent a new generation of antitumor drugs currently being tested in phase-I trials in patients with cancer. The present study reports the differential modulation of human immunocompetent cells in vitro by ALP. Serum-bound ALP effectively blocked the response of growth factor–dependent cells to interleukin-2 (IL-2), inhibited the cellular production and release of IL-2 and suppressed the comitogenic effect of interleukin-1 (IL-1) on mouse thymocytes. In contrast, ALP-primed, monocyte-derived macrophages (MO) lost their ability to release IL-1 in response to stimuli like lipopolysaccharides (LPS) during terminal maturation from monocytes. Supernatants from ALP-primed, LPS-induced MO possessed costimulatory as well as direct mitogenic activity. Neither ALP alone nor ALP-conditioned MO supernatants stimulated mouse thymocytes. Priming of MO by ET-18-OH, an ALP molecule not substituted in the *sn*-2 position, occurred at concentrations 4- to 16-fold higher than the most active compounds ET-18-OCH$_3$ and the thioether analogue BM 41.440. ALP also primed MO for subsequent activation of tumor cytotoxicity by LPS and interferon-gamma.

IL-1 has multiple biological activities in common with ALP, and it may mediate antitumor activity and other ALP effects in vivo. The ability of ALP to induce differential immunomodulation, as demonstrated in this study, may make ALP worthy of study for the therapy of both autoimmune and neoplastic disease.
Lipids 22, 836–841 (1987).

Synthetic alkyl analogues of natural 2-lysophosphatidylcholine (LPC) are a new class of antitumor agents currently being tested in phase-I trials in patients with tumors (1). In animal models, demonstration of a tumor-therapeutic activity requires both a low tumor burden and long-term application of rather small doses. In addition, ALP are anticarcinogenic (2) and protect from radiation damage (3). Despite well-documented in vivo effects, their underlying mechanisms of action are still unclear. Activation of macrophages to cytotoxicity (4), induction of cell differentiation (5), inhibition of tumor cell invasion (6) and a direct destructive effect on cells selective for neoplastic cells (1–9) have all been proposed. We previously reported on the interference of alkyl lysophospholipids (ALP) with normal lymphocyte transformation (10), and we now present data proving a differential effect of ALP on the production and biological activity of interleukin-1 (IL-1) and interleukin-2 (IL-2). The current study demonstrates that ALP-primed human monocyte-derived macrophages (MO) respond to subsequent stimuli with the release of large quantities of IL-1-like activity. ALP simultaneously inhibit lymphocyte production of IL-2 and lymphocyte responses to both IL-1 and IL-2.

MATERIALS AND METHODS

Lysophospholipids. Natural 2-LPC was purchased from Sigma Chemical Co. (St. Louis, MO) and *rac*-1-octadecyl-2-methoxy-*sn*-glycero-3-phosphocholine (ET-18-OCH$_3$) from Medmark Chemicals (Grünwald b. München, FRG). *Rac*-1-octadecyl-*sn*-glycero-3-phosphocholine (ET-18-OH) was provided by H. U. Weltzien (Max Planck Institut, Freiburg, FRG); *rac*-1-mercapto-hexadecyl-2-methoxymethyl-*sn*-glycero-3-phosphocholine (BM 41.440) was a gift from W. Pahlke (C. F. Boehringer Mannheim GmbH, Mannheim, FRG).

Cells. Peripheral blood leukocytes were separated from buffy coat preparations of healthy blood donors by density gradient centrifugation on Ficoll-Hypaque. Monocytes were isolated from other mononuclear cells (MNC) by adherence to plastic petri dishes (60 min at 5×10^6 MNC per ml RPMI1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, antibiotics and 15% fetal calf serum [FCS]), cultured overnight in RPMI1640 plus 10% human AB-group serum and recovered by vigorous pipetting at 4 C. Monocytes were greater than 90% pure as estimated by cytochemistry and antigen analysis (11). They were cultured in suspension at 3×10^5/ml RPMI1640 plus 10% AB-serum on hydrophobic Teflon foils for various time periods (12) and subjected to further experimentation at their sequential stages of differentiation. MO phenotype was determined to judge the stage of maturation (11), which was completed after 10 days in culture, after which the cells were termed mature monocyte-derived MO (TMO).

MLA144 cells (a T-cell line of gibbon origin, ref. 13) were provided by H. Rabin (National Cancer Institute, Frederick, MD), maintained in RPMI1640 plus 10% FCS and subcultured every 3–4 days.

Activation of monocyte MO and production of supernatants. Monocyte MO were cultured at 5×10^4 per 0.2 ml RPMI1640 plus 30% FCS for 24 hr with and without ALP. The medium was then replaced with fresh RPMI1640 plus 30% FCS, the cells were incubated for 30 min, and 10 μg/ml lipopolysaccharides (LPS, Salmonella abortus equi M6; donated by Dr. Galanos, Max Planck Institut, Freiburg, FRG) was added in RPMI1640 plus 10% FCS. Supernatants were collected after 24 hr, centrifuged at 200 g for 10 min and stored at 4 C.

Thymocyte proliferation assay (IL-1). Thymocytes prepared from 3- to 4-week-old C3H/HeJ mice (Bonholtgard, 868 Ry, Denmark) were cultured in triplicate at 5×10^6 cells/ml RPMI1640 plus 10% FCS with and without 1:200 diluted phytohemagglutinin (PHA) (Gibco, Grand Island, NY) in a final volume of 0.2 ml in flat-bottomed 96-well microplates containing 1:4 dilutions of test

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Abbreviations: ALP, alkyl lysophospholipids; IL-1 and IL-2, interleukin-1 and -2; MO, macrophages; MNC, mononuclear cells; TMO, mature macrophages; LPS, lipopolysaccharides; PHA, phytohemagglutinin.

supernatants. The cultures were incubated for three days before they were pulsed for 6 hr with 0.2 μCi ^{3}H-thymidine (sp act 28 Ci/mmol; Amersham and Buchler, Braunschweig, FRG) and subsequently harvested. IL-1 activity is expressed as counts/min (cpm). In some experiments, thymocytes were cultured with ALP alone or with and without ALP in the presence of recombinant human interleukin-1-alpha (Hoffman-LaRoche, Nutley, NJ) at a 1:7,500 dilution.

Bioassay for interleukin-2. IL-2-dependent mouse cells (HT-2, gift from Dr. Larrick, Cetus Co., Emeryville, FL) were passaged three times a week in an IL-2-containing medium conditioned for 48 hr by human blood MNC stimulated with 1:50 diluted PHA, 2 μg/ml concanavalin A and irradiated allogeneic lymphoblastoid cells in a ratio of 1:1. HT-2 cells were taken for the IL-2 assay after two days of last passaging, washed twice in serum-free medium and cultured with the 1:2 diluted test supernatants for 24 hr before the 6-hr ^{3}H-thymidine incorporation was measured.

Tumor-cytostasis assay (14). Monocyte-derived TMO were seeded at 5 × 10^4/0.2 ml RPMI1640 plus 30% FCS and cultured for 24 hr with or without 16 μg/ml ET-18-OCH$_3$. MO were then incubated twice with fresh complete medium for 30 min each time and subsequently cultured with serum-free RPMI1640 (containing 0.1 mg/ml lactalbuminhydrolysate, 0.2 mg/ml fetuin and 0.15 U/ml swine insulin) in the presence of LPS or recombinant human interferon-gamma (rIFNg, Biogen, Geneva, Switzerland) for 24 hr. MO monolayers were then rinsed twice with warm serum-free medium before 10^4 U937 cells (K. Nielsson, Uppsala, Sweden) were added. After coincubation of MO and targets for another 48 hr, the cultures were pipetted to bring target cells into suspension, 0.1 ml aliquots were transferred to a new microplate and ^{3}H-thymidine uptake was measured with a 6-hr pulse. Activation indices were calculated by dividing cpm values of target cells after coincubation with control MO vs cpm values of target cells after coculture with activated MO.

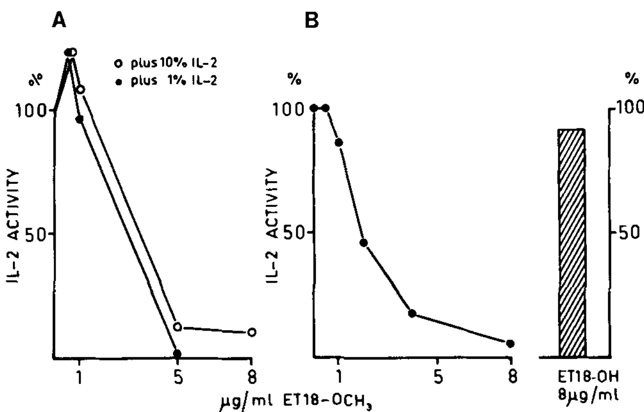


FIG. 1. Inhibition of (A) response to and (B) activity of interleukin-2 (IL-2) by alkyl lysophospholipids (ALP). HT-2 cells were cultured with IL-2 standard preparations in the presence of ET-18-OCH$_3$. After 24 hr, the 6-hr thymidine uptake was measured; results are expressed as percentage of control cultures without ET-18-OCH$_3$ (part A). In part B, MLA144 cells were cultured with ET-18-OCH$_3$ or ET-18-OH for 24 hr; fresh medium was added and supernatant collected after another 24-hr culture period. IL-2 activity was assessed therein as described above and is expressed as percentage of control supernatants from cells cultured without ALP, mean of triplicate values, S.D. less than 15%.

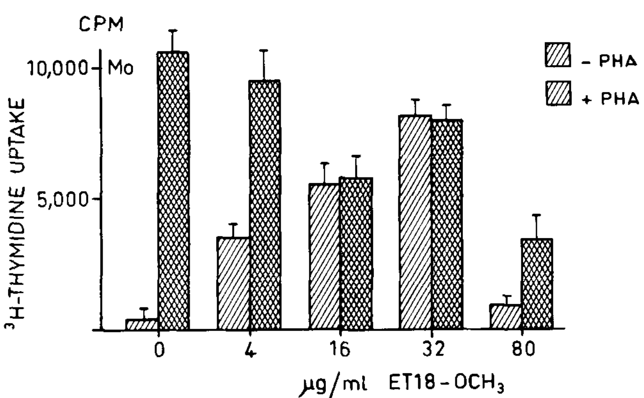


FIG. 2. Inhibition of the comitogenic (interleukin-1-like) and generation of a mitogenic activity in lipopolysaccharides stimulated blood monocytes after preculture with alkyl lysophospholipids (ALP). Blood monocytes were separated by adherence, cultured overnight and incubated with ALP for 24 hr. Then the medium was changed, cells were rinsed twice and 10 μg/ml LPS was added for another 24 hr before supernatants were collected and tested for their ability to be directly stimulatory to mouse thymocytes or indirectly in the presence of PHA. Results are given as counts/min, mean of triplicate values.

RESULTS

Based on our earlier findings that ALP interfere with normal lymphocyte transformation and inhibit growth of activated lymphoblasts (10), we started an investigation into the immunomodulatory effects of ALP in vitro by looking at the response of IL-2-dependent cells in the presence of ALP. As shown in Figure 1A, ET-18-OCH$_3$ inhibited IL-2 response of HT-2 cells at concentrations above 2 μg/ml, whereas lower doses had a minor, reproducible stimulatory effect. The production of IL-2 was also inhibited within a similar dose range of ET-18-OCH$_3$ (Fig. 1B). Both effects were seen only with those analogues of 2-LPC that are modified in *sn*-1 and *sn*-2, whereas the ether analogue ET-18-OH had no effect. In the presence of ALP, the response of normal lymphoblasts to IL-2-containing supernatants is suppressed similarly, as is the release of IL-2 (data not shown).

Peripheral blood monocytes respond to activation by LPS with the release of the lymphocyte-activating factor termed IL-1 (15). We measured IL-1 in MO supernatant by its ability to act on C3H/HeJ mouse thymocytes either directly or in the presence of the mitogen PHA. If monocytes were first incubated for 24 hr with ET-18-OCH$_3$, they responded to subsequent LPS stimulation with the release of decreasing amounts of costimulatory activity (Fig. 2). However, a dose-dependent increase of a direct mitogenic activity was found in LPS-stimulated, ALP-preincubated monocyte supernatant.

When blood monocytes are cultured on hydrophobic Teflon foils in the presence of human AB-group serum, they differentiate into mature MO-type cells (for details, see refs. 11 and 12). During this in vitro maturation, they rapidly lose their ability to respond to LPS stimulation with IL-1 release (Fig. 3), as has been shown by others using different culture systems (16). However, if these

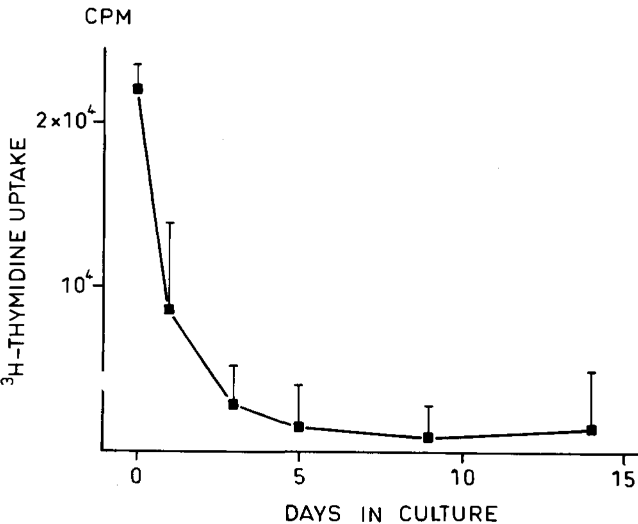


FIG. 3. Release of interleukin-1 (IL-1) activity by human monocytes/macrophages at different stages of in vitro maturation. Monocytes as well as monocyte-derived macrophages obtained after various days in culture were cultured with 10 μg/ml lipopolysaccharides for 24 hr. Thereafter, supernatants were tested for IL-1-like activity on mouse thymocytes. Data are mean of triplicates from three separate experiments.

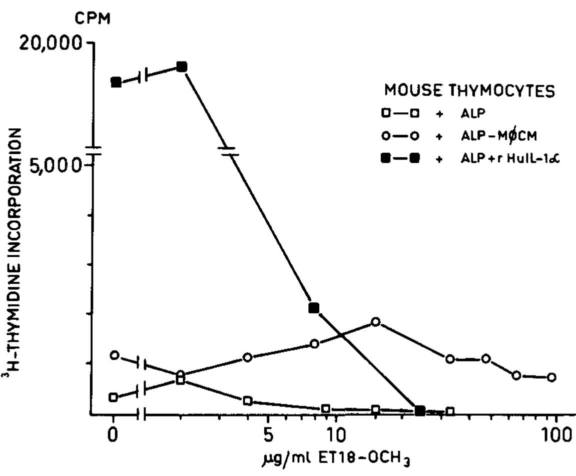


FIG. 5. Effect of alkyl lysophospholipids (ALP) and ALP-conditioned macrophage (MO) supernatants on mouse thymocytes and the comitogenicity of interleukin-1 (IL-1). Mouse thymocytes were cultured with suboptimal PHA concentrations with ALP and ALP-conditioned MO supernatants, respectively, either alone or in the presence of recombinant human IL-1-alpha at a dilution of 1:7,500 for 3 days before thymidine uptake was measured. Data are expressed as cpm, mean of triplicate values, S.D. less than 15%.

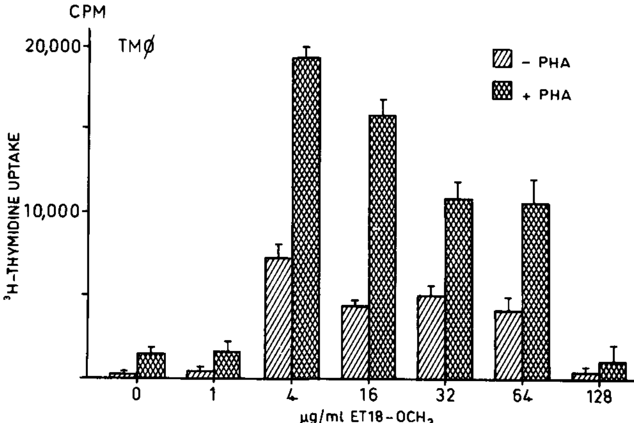


FIG. 4. Generation of interleukin-1 (IL-1)-like activity in lipopolysaccharide (LPS)-stimulated monocyte-derived macrophages (MO) after induction with alkyl lysophospholipids. Teflon culture-derived mature MO were incubated with various concentrations of ET-18-OCH$_3$ for 24 hr and rinsed twice with fresh medium before 10 μg/ml lipopolysaccharides were added. After another 24-hr culture period, supernatants were collected and tested. For further details, see legend to Fig. 2.

monocyte-derived TMO were first cultured with ALP for 24 hr, they retain their ability to mount an effective response to LPS stimulation and secreted high amounts of IL-1-like material in relation to the ALP concentrations used (Fig. 4). Again, the MO supernatant transformed thymocytes directly but also effectively potentiated the mitogenicity of suboptimal PHA concentrations. This dose-dependent "priming" activity of ALP on TMO is still seen at concentrations in the near-toxic range: only about 50% of the MO exclude trypan blue after a 24-hr culture with 64 μg/ml ALP (data not shown).

TMO cultured with ALP alone without a subsequent second stimulus did not release any IL-like material. In addition, ALP itself had no stimulatory effect in the comitogenic assay. In contrast, ALP effectively blocked the thymocyte response to recombinant IL-1-alpha (Fig. 5).

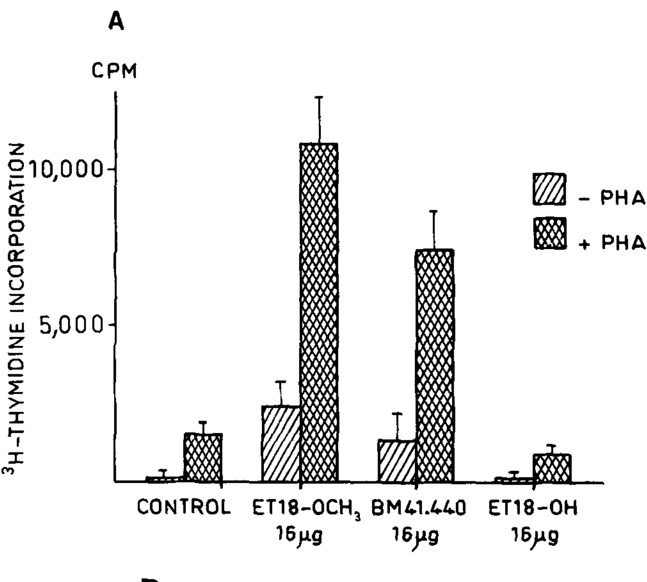
Other ether analogues of 2-LPC also preactivated TMO when modified in both the *sn*-1 and *sn*-2 positions (like the thioether analogue BM 41.440, Fig. 6A). However, ET-18-OH is as effective in priming MO as the most active analogue ET-18-OCH$_3$, although requiring a 4- to 16-fold higher concentration (Fig. 6B).

TMO maintained their state of primed activity for about 24 hr after removal of the ALP. In Figure 7, results of a typical experiment are shown where TMO were cultured for 24 hr with or without 16 μg/ml ET-18-OCH$_3$, and at various times after removal of the ALP were recultured in serum-free medium stimulated with LPS.

As IL-1 secretion is only one parameter indicative of an activated state of MO, we also evaluated the activity of ALP-primed TMO as tumor-cytotoxic effector cells. As shown in Figure 8, pretreatment of ALP augmented the response of TMO to activation for tumor cytotoxicity. As demonstrated, other MO activators like interferon-gamma can also act as the second stimulus, thus establishing the preactivated state of ALP-primed MO. In these experiments, ALP alone did not activate MO for tumor cytotoxicity (data not shown).

DISCUSSION

Synthetic ALP were the first reported molecularly defined lipid mediators that possess immunomodulatory as well as other biological activities (17). A decade later, the description of the ALP structure of platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphatidylcholine) defined a new class of naturally occurring ether lipids with hormone-like activity (18–20).



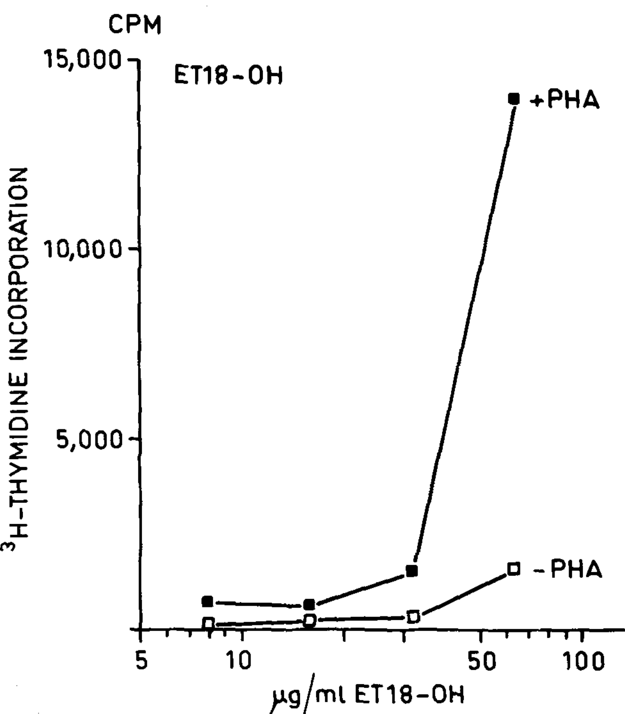


FIG. 6. Priming of monocyte-derived macrophages (MO) by alkyl lysophospholipids (ALP): dependence on the molecular structure of the ALP. Teflon culture-derived mature MO were incubated 24 hr with various ALP compounds at 16 µg/ml (A) and with ET-18-OH at increasing concentrations (B) before lipopolysaccharides were added. Supernatants were collected after 24 hr and tested on mouse thymocytes. Data are indicated in part A and are mean of triplicates for part B; S.D. less than 15%.

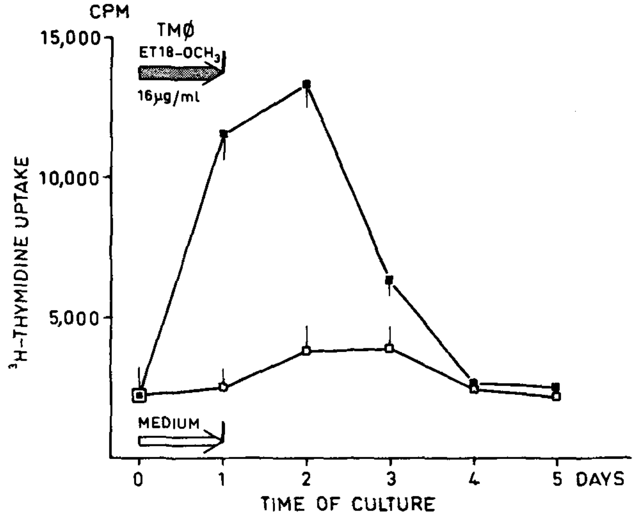


FIG. 7. Duration of the alkyl lysophospholipid (ALP)-induced state of primed activity in monocyte-derived macrophages (MO). Teflon culture-derived mature MO were incubated with medium alone or with 16 µg/ml ET-18-OCH₃. Lipopolysaccharide was then added at 10 µg/ml for 24 hr either directly thereafter or following cultivation of the MO in serum-free RPMI1640 for various time periods. For further details, see legend to Fig. 2.

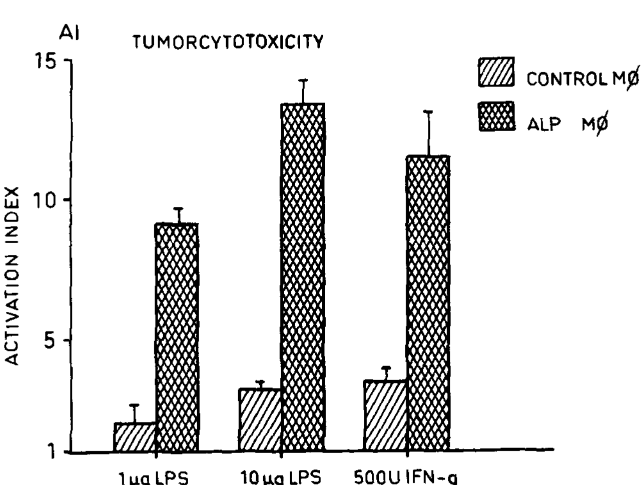


FIG. 8. Enhancement of tumor-cytotoxic effector cell activation by lipopolysaccharides (LPS) and interferon-gamma after precultivation of macrophages (MO) with alkyl lysophospholipids (ALP). Mature MO (TMO) were sequentially cultured in microplates with 16 µg/ml ET-18-OCH₃ for 24 hr and LPS or interferon-gamma (also 24 hr) before U937 tumor cells were added and cocultured with the mature MO (TMO) for 48 hr. Thereafter, the microcultures were pipetted, and ^{3}H-thymidine uptake into 0.1 ml aliquots was measured in a separate microplate. Results are expressed as activation indices calculated by dividing cpm of tumor cells after coculture with control TMO vs cpm of tumor cells after coculture with activated TMO, mean of triplicate values.

The results of the present study provide evidence for a novel function of ether lipids, i.e., the differential manipulation of immune responses in vitro. ALP inhibited lymphocyte response to IL-2 and rIL-1-alpha and suppressed the production of IL-2 in competent cells. These effects may be related to our recent discovery that mitogen-stimulated lymphoblasts not only ceased to grow but rapidly died in the presence of ALP. Small resting lymphocytes are unaffected by ALP (10), while the activation of lymphocytes by a variety of stimuli is supressed by ALP (10). In contrast, ALP render monocyte-derived MO responsive to subsequent activating stimuli. Only ALP-"primed" MO, but not untreated control MO, could be stimulated by LPS to release high amounts of

a lymphocyte-activating factor activity. In addition to its costimulatory effect, this factor was also directly mitogenic in the mouse thymocyte assay. As purified and recombinant human IL-1 preparations do not activate lymphocytes directly (21), the activity found in the ALP-primed, LPS-stimulated MO supernatant should be termed "IL-1-like material," which must contain more than the single "mature" polypeptide (17,500 mol wt), defined to be the extracellular secreted form of IL-1 (22). Biological activity has been described also for small peptide fragments (23), and an intracellular, nonsecreted precursor molecule with a higher molecular weight (31,000 mol wt) has been shown to be directly mitogenic (24). ALP primed MO at concentrations in the toxic range. It is thus possible that intracellular IL-1 precursors were released and were, in part, responsible for the observed effect of ALP-primed MO. High performance liquid chromatography experiments are in progress to further elucidate the molecular nature of this IL-like activity.

No IL-1-like material was induced by ALP alone. The molecular mechanism by which ALP induce MO preactivation is not understood. Possible explanations may be linked to the ALP-induced inhibition of key phospholipid—metabolizing enzymes (25), which provide arachidonic acid for the biogenesis of leukotrienes and prostaglandins. Interestingly, indomethacin, which like ALP inhibits arachidonic acid metabolism, also enhanced IL-1 production (26). There may well also be an interaction between lipid and peptide mediator synthesis (27).

ALP represent a new generation of potent antitumor drugs (1). However, the clinical tumor-therapeutic potential of ALP is, to say the least, incompletely understood. Apart from a direct cytotoxic effect on malignant cells (7-9) and the inhibition of tumor cell invasion (6), these compounds induce various subtle changes in cell biology (e.g., activation of cytotoxic effector cell function [4] and induction of cellular differentiation [5]). ALP are also highly active adjuvants (17) in both in vivo and in vitro cell cooperation systems (28). ALP also protect animals from radiation damage by mechanisms as yet unexplained (3).

Multiple biological activities have also been ascribed to IL-1 (21). Some striking similarities exist between the effects of ALP and IL-1 in vitro and after administration in vivo; for example, IL-1 also is cytostatic for tumor cells in vitro (29,30), is a radioprotector (31), activates MO for cytotoxicity (32) and induces monophasic fever (21) and chills, which we observed in two tumor patients after high-dose intravenous application of ALP (personal observation). IL-1 secretion from mature tissue MO in synergism with other activating signals present in the microenvironment may be a mediator for many of the effects observed after ALP administration in vivo. In addition, the effects of ALP on IL-1-producing cells other than monocyte MO (15) need to be evaluated.

ALP preactivate MO not only for IL-1 release but also for other effector cell functions of activated cells (e.g., tumor cytotoxicity, prostaglandin E_2 secretion (Andreesen and Steinhauer [manuscript in preparation]) and probably others still to be looked at.

In conclusion, the study demonstrates a new activity of ALP. This "immunomodulatory" activity may be an important aspect of its tumor-therapeutic role and also its interaction with immunocompetent cells. The differential action of ALP on the immune system (summarized in Fig. 9) with MO activation and suppression of lymphocyte function could indicate a potential for beneficial effects of ALP in autoimmune diseases and in the acquired immunodeficiency syndrome (AIDS).

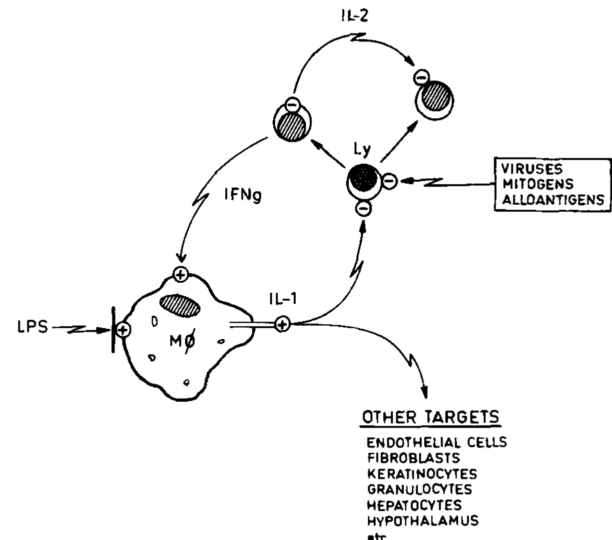


FIG. 9. Differential effects of alkyl lysophospholipids (ALP) on human immunocompetent cells in vitro. ALP restore the capacity of mature macrophages (MO) to respond to lipopolysaccharides (LPS) with the release of interleukin-1 (IL-1)-like activity and also augment activation of MO cytotoxicity by interferon-gamma and LPS. ALP inhibit lymphocyte response to IL-1, interleukin-2 (IL-2) and in general transforming stimuli. ALP, in addition, block IL-2 synthesis. IL-1 seems to be directed to other, non-lymphocyte, targets.

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A Metabolite of an Antineoplastic Ether Phospholipid May Inhibit Transmembrane Signalling Via Protein Kinase C[1]

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In our search for the mechanisms by which the drug 1-*O*-alkyl-2-*O*-methylglycero-3-phosphocholine (AMG-PC) inhibits tumor growth and metastasis, we have detected a metabolite, 1-*O*-alkyl-2-*O*-methylglycerol (AMG), in membranes of MO_4 mouse fibrosarcoma cells grown in the presence of the drug. Synthetic AMG inhibited the activation of highly purified human protein kinase C by diacylglycerol in the presence of phosphatidylserine. Furthermore, AMG also inhibited the receptor-specific binding of ^{3}H-phorbol-12,13-dibutyrate to human HL-60 promyeloid leukemia cells in a dose-dependent fashion. AMG-PC was not effective or much less so in these assays. We suggest that interaction of the metabolite AMG with protein kinase C may inhibit stimulus-response coupling in tumor cells and may thus potentially contribute to the mechanism by which AMG-PC exerts its anticancer activities.

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The drug 1-*O*-alkyl-2-*O*-methylglycero-3-phosphocholine (AMG-PC) has been shown to specifically inhibit the growth of tumor cells in vitro and in vivo, to inhibit tumor cell invasion and metastasis and to enhance the tumoricidal capacity of macrophages (reviewed in ref. 1). The mechanisms involved in these actions are unknown. Besides the possibility that accumulation of the drug as such in cellular membranes (2) may alter their structure and functioning, possible metabolic products of the drug should also be considered. Since AMG-PC is not a substrate for the alkyl cleavage enzyme (3), possible metabolites may only be formed via enzymatic cleavage of the phosphate bond.

In the present report, we show that the metabolite 1-*O*-alkyl-2-*O*-methylglycerol (AMG) is detectable in membranes of tumor cells grown in the presence of AMG-PC. Given the structural analogy of this metabolite to 1,2-diacylglycerol, a natural activator of protein kinase C (PKC) (4), we have investigated the possibility that AMG interacts with this key enzyme in cell signal transduction. First, we demonstrate that AMG inhibits the diacylglycerol-stimulated activity of a highly purified preparation of human PKC. Second, we show that AMG inhibits the binding of a biologically active phorbol ester to its receptor (PKC) on human HL-60 cells.

MATERIALS AND METHODS

Chemicals. The AMG-PC analogs racemic 1-*O*-octadecyl- and 1-*O*-hexadecyl-2-*O*-methylglycero-3-phosphocholine as well as 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG) were purchased from Bachem AG (Bubendorf, Switzerland). 1,2-Dioctanoyl-*sn*-glycerol (diC_8) was obtained from Avanti Polar Lipids (Birmingham, AL). Phosphatidylserine (PS; from bovine brain), 1,2-dioleoyl-*rac*-glycerol (diolein), leupeptin, phenylmethylsulphonyl fluoride (PMSF), soybean trypsin inhibitor, aprotinin, histone IIIS (from calf thymus) and adenosine 5′-triphosphate (ATP) were obtained from Sigma Chemical (St. Louis, MO). ^{32}P-ATP (3 Ci/μmol) and ^{3}H-phorbol-12,13-dibutyrate (PDBu, 12.5 Ci/mmol) were from New England Nuclear (Boston, MA), and cholesterol and ethylene-glycol-bis(beta-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA) from BDH (Poole, U.K.). All chemicals were of analytical grade.

Cells. MO_4 cells are virally transformed C_3H mouse fibroblastic cells that are invasive in vitro (5) and produce invasive and metastasizing fibrosarcomas in syngeneic mice (6). They were cultured in minimum Eagle's medium (Rega I; Gibco Europe, Paisley, U.K.) supplemented with 10% fetal calf serum and 0.05% glutamine. Cells were also grown in the presence of AMG-PC (octadecyl-type, 7 μg/ml culture medium), added 48 hr prior to membrane lipid analysis. The concentration of the drug used permitted growth to about 80% of that of control cells (5). Human HL-60 promyeloid leukemia cells (7) were maintained as a suspension culture in RPMI 1640 medium, supplemented with 20% fetal calf serum.

AMG detection in membranes. A crude membrane fraction from MO_4 cells was prepared by centrifugation of the low-speed supernatant of a cell homogenate at 105,000 × g for 1.5 hr, as described before (8). Lipids were extracted with chloroform/methanol (2:1, v/v) followed by partition according to Folch et al. (9) and were separated by thin layer chromatography (TLC) on precoated silica gel plates (Merck, Darmstadt, FRG) using hexane/ether/acetic acid (60:40:1.6, v/v). The spot corresponding to AMG was scratched off, extracted and trimethylsilylated with a mixture of pyridine-hexamethyldisilazane-trimethylchlorosilane (12:5:2, v/v/v; obtained from Pierce Chemical Co., Rockford, IL) for 2 hr at room temperature and analyzed by gas liquid chromatography according to Myher and Kuksis (10), using a 25 m CP-Sil 58 column. AMG was also enzymatically prepared from AMG-PC (octadecyl-type) using phospholipase C (20 U/ml) from *Clostridium perfringens* (obtained from Sigma) in 10 mM $CaCl_2$, 0.1 mM $ZnCl_2$, 20 mM Tris-HCl (pH 7.4) at 37 C for 15 hr. The AMG formed (in low yield) was separated from AMG-PC by TLC and trimethylsilylated

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Abbreviations: AMG-PC, 1-*O*-alkyl-2-*O*-methylglycero-3-phosphocholine; AMG, 1-*O*-alkyl-2-*O*-methylglycerol; PKC, protein kinase C; diC_8, 1,2-dioctanoyl-*sn*-glycerol; PS, phosphatidylserine; PMSF, phenylmethylsulfonyl fluoride; ATP, adenosine 5′-triphosphate; PDBu, ^{3}H-phorbol-12,13-dibutyrate; EGTA, ethylene-glycol-bis(beta-aminoethylether)-N,N,N′,N′-tetraacetic acid; TLC, thin layer chromatography.

as described above. The AMG thus prepared and that isolated from MO_4 cell membranes were identical, as unambiguously shown in Figure 1. AMG was quantitated by the detector response, using a known amount of heptadecanoic acid methyl ester as a reference.

Phorbol ester receptor assay. Binding of ^{3}H-PDBu to HL-60 cells in the presence or absence of inhibitors, supplied in dimethyl sulfoxide (0.5% final content), was performed by the method of Goodwin and Weinberg (11) in polypropylene tubes, using 5–10 × 10^5 cells and 20 nM ^{3}H-PDBu per assay (0.2 ml), unless otherwise stated. AMG and ^{3}H-PDBu were added simultaneously. Following incubation at room temperature for 30 min, excess cold assay buffer was added, and free and receptor-bound ^{3}H-PDBu were immediately separated by filtration through glass-fiber filters (Whatman GS/A). The filters were washed rapidly with excess buffer and prepared for liquid scintillation counting. The results are presented as specific binding, that is, the difference between ^{3}H-PDBu bound in the presence and absence of 20 μM unlabeled PDBu.

Purification of protein kinase C. Human PKC was isolated from a platelet/lymphocyte fraction of 50 buffy coats of donor blood. The purification procedure follows essentially the methods of Kikkawa et al. (12) and Girard et al. (13) and will be described in detail elsewhere. In brief, buffy coats were centrifuged (1300 × g, 8 min, 20 C) to remove granulocytes and erythrocytes. The rest of the procedure was performed at 0–4 C. Platelets and lymphocytes were spun down at 1700 × g (45 min) and taken up in 5 vol homogenization buffer containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA and protease inhibitors (leupeptin, trypsin inhibitor, PMSF and aprotinin). Cells were broken in a Omni homogenizer (Sorvall) and the 100,000 × g supernatant of the cell homogenate underwent three chromatographic steps: DEAE cellulose, Phenyl sepharose CL-4B and affinity column chromatography using phosphatidylserine and cholesterol in the column matrix (13). PKC was eluted from the affinity column with 10 ml of 5 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 5 mM 2-mercaptoethanol, 2 mM EGTA in a yield of about 50 μg protein. The PKC preparation thus obtained was 7,500-fold purified relative to the cytosolic fraction and showed one single band of 81 kD (see Fig. 2) upon SDS-polyacrylamide gel electrophoresis (14). The enzyme was stored in 40% sucrose at −70 C until use.

Enzyme assays. The activity of PKC in the presence or absence of stimulatory/inhibitory lipids was determined essentially according to Kikkawa et al. (15). The assays were performed in a 2 mM Ca^{2+}-EGTA buffer (16) yielding 10 μM free Ca^{2+} or in 2 mM EGTA. The reaction mixtures (60 μl final volume) furthermore contained 10 mM Mg^{2+}, 20 mM Tris-HCl (pH 7.5), 200 μg/ml histone IIIS, 20 μM leupeptin, 10 μM ^{32}P-ATP (0.5–1 μCi per assay) and PKC (10 μl preparation). Lipids were present in amounts indicated in the footnote of Table 1 and the legend of Figure 2. They were added as liposomes (10-μl samples), prepared by sonication for 1 min in a Branson Sonifier equipped with a standard probe (50 W). Incubations were started by addition of ATP and performed for 5 min at 30 C. The reaction was stopped by precipitation on Whatman paper filters (3 Chr) in cold 10% trichloroacetic acid plus 10 mM K_2HPO_4. The filters were extensively washed and prepared for liquid scintillation counting.

RESULTS AND DISCUSSION

In a previous study (5), it was demonstrated that the anticancer drug AMG-PC inhibits the invasion of MO_4 fibrosarcoma cells into normal embryonic chick heart tissue, under conditions where growth of the tumor cells in tissue culture was only minimally affected. In the companion article (2), we have shown that under these conditions AMG-PC is accumulated in the tumor cell membranes, predominantly in the plasma membrane: MO_4 cells grown in the presence of 5–10 μg/ml AMG-PC for 48 hr incorporated the drug to about 10% of the total phospholipids (measured in purified plasma membranes). Figure 1 shows

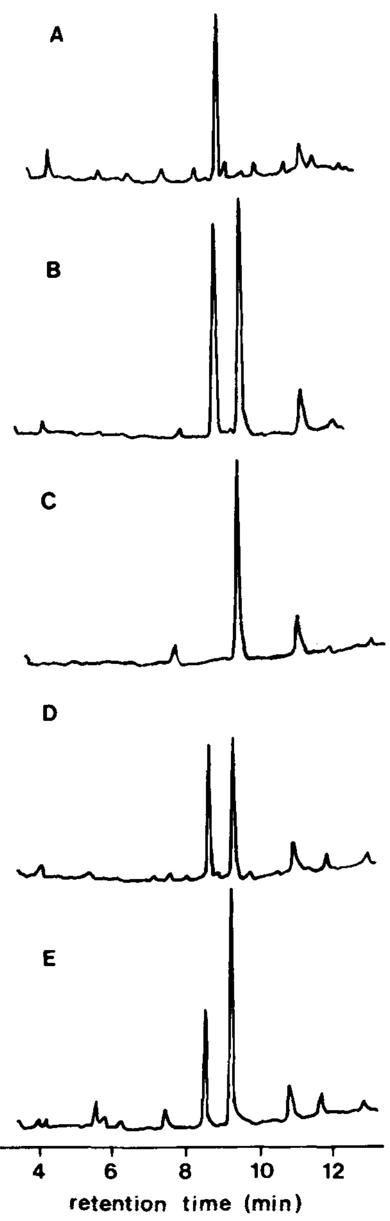

FIG. 1. Detection of 1-*O*-octadecyl-2-*O*-methylglycerol (AMG) in membranes from MO_4 cells grown in the presence of 1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine (AMG-PC). Gas liquid chromatography of trimethylsilyl derivatives. The first peak, at a retention of 8.7 min, was not identified. (A) Control cells; (B) cells grown with AMG-PC; (C) AMG prepared from AMG-PC (octadecyl-type) using phospholipase C from *Cl. perfringens*; (D) C added to A; (E) C added to B.

that the incorporated drug was partly metabolized to AMG, presumably by a phospholipase C-like enzyme that is able to split off or exchange the phosphocholine moiety. The gas chromatogram (Fig. 1B), pertaining to cells grown with AMG-PC, shows two main peaks (retention times 8.7 and 9.3 min), the first of which is an unidentified compound that was also found in the control cells (Fig. 1A), while the second peak was identified as AMG. The amount of AMG detected in the membrane preparation corresponded roughly to 1 μg (or 3 nmol) per 10^8 cells. It should be noted that this is a steady-state value. Nothing is known about any possible further metabolism of AMG, such as conversion to a phosphatidic acid analogue or degradation by an alkyl cleavage enzyme (3).

Many cellular activities, including proliferation, differentiation and secretion in endocrine and exocrine systems, depend on transmembrane stimulus-response coupling that may be mediated by PKC (4). Although not yet established, it is not unlikely that this key enzyme in signal transduction is also involved in the mechanism of (tumor) cell adhesion and invasion, possibly through specific phosphorylation of cytoskeleton-associated proteins (4). PKC is thought to be physiologically activated by diacylglycerol derived from receptor-mediated phosphatidylinositol hydrolysis and is the receptor for biologically active phorbol esters, such as PDBu (4). In the following experiments, we have studied the interaction of AMG-PC and AMG with PKC, in both an enzymatic assay and a PDBu-receptor assay.

We have purified human PKC to apparent homogeneity (Fig. 2, right) and used this preparation to study the lipid dependency of the enzyme in the presence of 10 μM Ca^{2+} and in submicromolar Ca^{2+} (excess EGTA) (Table 1). In agreement with literature data (4,15,17), PS was found to be necessary to achieve substantial activation. At submicromolar free Ca^{2+}, like under physiological conditions in the resting cell, only diacylglycerol (diolein) is able to activate PKC in the presence of PS (six- to eightfold stimulation, relative to the basal level with PS only). Diolein alone had no effect (not shown here). At 10 μM Ca^{2+}, the PS-dependent basal activity is much higher, and diolein is not able to increase this activity any further (exp. 2) or more than 30% (exp. 1). Neither AMG-PC nor AMG activate PKC by themselves or in the presence of PS. Rather, they inhibit the basal activities in the presence of PS some 10–20%. The largest inhibitory effect of AMG was found specifically on the diacylglycerol stimulation of the enzyme, precisely the activity that is physiologically relevant and that is generated in the cell upon receptor stimulation. This inhibitory effect of AMG, shown in Figure 2, was dose-dependent and was present in excess EGTA as well as in 10 μM Ca^{2+} (the latter being relevant only in experiment 1, where diolein was indeed stimulating the PS-dependent activity of the enzyme; see Table 1). We have no clear explanation for the variability generally found in the degree of diolein stimulation of the PS-dependent enzyme activity among individual experiments. In contrast to AMG, AMG-PC does not have a significant effect on the diolein-stimulated PKC activity (Fig. 2). As noted above, the drug only inhibits the basal PS-dependent activities somewhat, confirming to a certain extent results of Helfman et al. (18). These authors, however, used only a very crude PKC preparation, unphysiologically high Ca^{2+} concentrations and no diacylglycerol.

TABLE 1

Lipids Dependency of the Activity of Purified Human Protein Kinase C (PKC) toward Histone as a Substrate

	PKC activity (pmol ^{32}P incorporated per min per 10 μl PKC preparation)[a]			
	2 mM EGTA		10 μM Ca^{2+}	
Lipids in assay[b]	Exp. 1	Exp. 2	Exp. 1	Exp. 2
PS	0.4	0.5	5.0	6.2
PS + diolein	3.1	2.9	6.4	6.4
PS + AMG		0.4		5.4
PS + AMG-PC		0.4		5.0
AMG		0.4		0.3
AMG-PC		0.4		0.4
No lipid	0.3	0.3	0.3	0.2

PS, phosphatidylserine; AMG, 1-*O*-alkyl-2-*O*-methylglycerol; AMG-PC, 1-*O*-alkyl-2-*O*-methylglycero-3-phosphocholine.

[a]Data are means of triplicates, with SD < 12%.

[b]PS, diolein, AMG and AMG-PC: 100, 5, 13.3 and 20 μg/ml assay mixture, respectively.

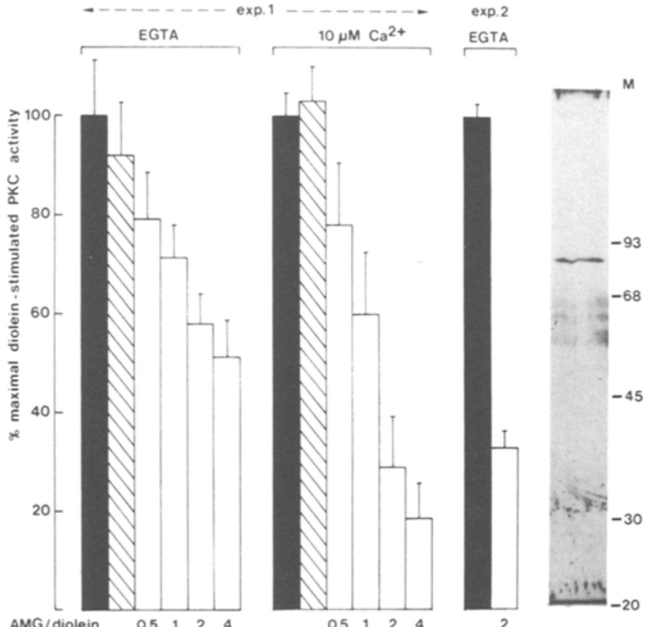

FIG. 2. Effect of 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (AMG-PC) and 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG) on the diacylglycerol-stimulated, phosphatidylserine (PS)-dependent activity of purified human protein kinase C (PKC) (basal PS-dependent activity, given in Table 1, subtracted) in the presence of 2 mM EGTA or 10 μM Ca^{2+}, as indicated. Solid columns, controls (100%), containing only PS and diolein, 100 and 5 μg/ml assay mixture, respectively; hatched columns, AMG-PC added in a ratio (w/w) AMG-PC/diolein = 2; open columns, AMG added in the ratios (w/w) indicated. Data are means of triplicate values, with SD as indicated. Experiments 1 and 2 correspond to those indicated in Table 1. Right panel: polyacrylamide gel showing the purity of the PKC preparation (from human lymphocytes/platelets) used in the present experiments. The silver-stained gel shows one band at 81 kD. Positions of authentic marker proteins (M) are indicated.

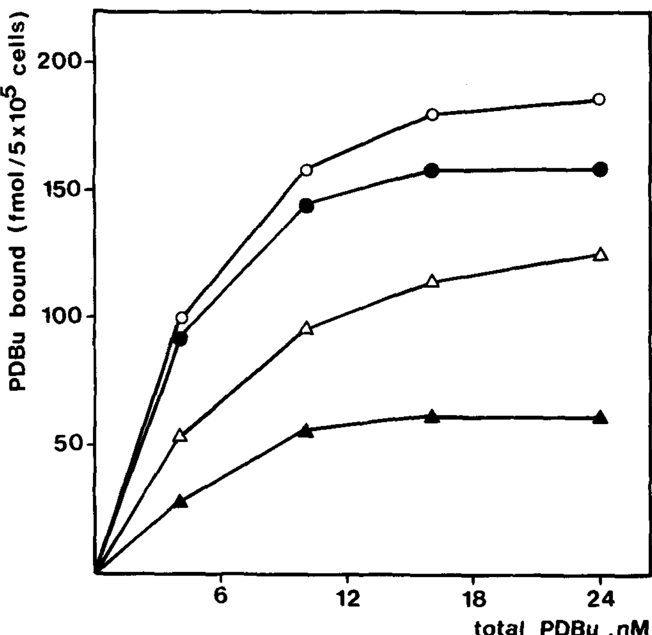

FIG. 3. Binding of ^{3}H-phorbol-12,13-dibutyrate (^{3}H-PDBu) to intact HL-60 cells in the presence of various concentrations of 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG) as a function of the total ^{3}H-PDBu concentration in the medium. ○, Control cells, without AMG; ●, 25 μM; △, 100 μM; ▲, 200 μM AMG. A representative experiment is shown where data points are means of triplicates (SD < 10%).

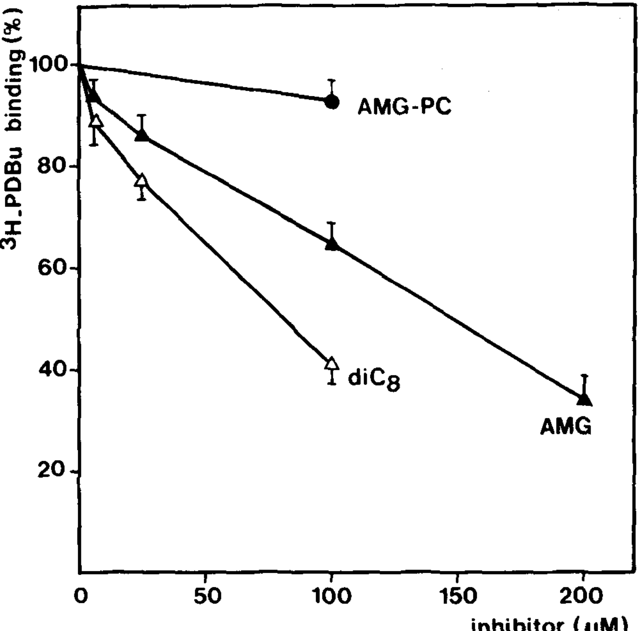

FIG. 4. Inhibition of ^{3}H-phorbol-12,13-dibutyrate (^{3}H-PDBu) binding to intact HL-60 cells by various concentrations of 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (AMG-PC) (●), 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG) (▲), 1,2-dioctanoyl-*sn*-glycerol (diC_8) (△), in the presence of 20 nM ^{3}H-PDBu. Data points are means of triplicates, with SD as indicated.

A substantial inhibition of the diacylglycerol-stimulated PKC activity, as we have found with AMG, should also be demonstrable in more complicated biological systems, such as PKC-mediated differentiation of HL-60 cells. As a first approach, we have studied in this cell system the effect of AMG on the specific binding of a biologically active phorbol ester, ^{3}H-PDBu, to its receptor (PKC). As shown in Figure 3, this binding is indeed inhibited by AMG in a dose-dependent fashion. At 20 nM PDBu, about 70% inhibition was reached with 200 μM AMG. Under these conditions, we also determined the inhibitory capacity of AMG-PC and diC_8, a membrane-permeable synthetic diacylglycerol, known to be a potent activator of PKC (19,20). Figure 4 shows that ^{3}H-PDBu binding is only minimally inhibited by AMG-PC, whereas diC_8 inhibits this binding twice as much as does AMG. However, if one takes into account that generally only the 1,2-*sn*-enantiomers of diacylglycerol (analogs) are biologically active (17,20) and that AMG is racemic, the inhibitory capacities of AMG and diC_8 may be the same. The concentration in the medium at which these compounds inhibit PDBu binding may seem high, but is, of course, dependent on their extent of incorporation (partitioning) into the apolar region of the cell membrane. This degree of uptake in the membrane is as yet unknown, but may likely be very low. As noted, we have found a steady-state content of about 1 μg (3 nmol) AMG per 10^8 MO_4 cells cultured in the presence of a dose of AMG-PC that only minimally inhibited cell growth. This cellular content of AMG is of the same order of magnitude as the amount of diacylglycerol generated in other cell types upon receptor stimulation (21). This notion together with the data on AMG inhibition of the diacylglycerol-stimulated PKC activity (Fig. 2) could indicate that the formation of AMG from AMG-PC in the cell membrane is physiologically relevant.

In conclusion, the anticancer drug AMG-PC accumulating in tumor cell membranes has a relatively small inhibitory effect on the enzymatic and phorbol ester receptor activities of PKC. However, its metabolite, AMG, detectable in the membranes of tumor cells grown in the presence of the drug, has much larger effects. It inhibits dose-dependently both the binding of PDBu to its receptor and the diacylglycerol-stimulated activity of PKC. The latter effect of AMG on this key enzyme in transmembrane signalling may potentially contribute to the mechanism by which AMG-PC exerts its anticancer activities. Whether this is indeed of physiological relevance remains to be further investigated.

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Effect of Lipid Derivatives on Invasion In Vitro and on Surface Glycoproteins of Three Rodent Cell Types[1]

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The antiinvasive activity on MO_4 mouse cells of the following lipid derivatives was tested in vitro: an alkyl-lysophospholipid derivative (ET-18-OCH_3), a thioether-phospholipid derivative (BM 41.440), an alkyl-linked lipoidal amine (CP-46,665) and a naturally occurring ester-linked phospholipid (2-LPC). In this test, BM 41.440 had the same antiinvasive potency as ET-18-OCH_3, whereas CP-46,665 and 2-LPC had no effect on invasion. Comparison of the antiinvasive effect of ET-18-OCH_3 on three types of cells showed the following ranking: 12R1C-RK rat kidney adenovirus type 12 transfected cells > MO_4 mouse cells > LLC-H61 Lewis lung carcinoma cells. This ranking was not reflected in ET-18-OCH_3-induced changes of cell surface exposed glycopeptides derived from the three types of cells metabolically labeled with radioactive fucose. The present and previous experiments suggested that changes in invasion caused by lipid derivatives depended upon relative cell surface fucosyl-glycopeptide alterations in both the invasive cells and the normal tissue.

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The alkyl-lysophospholipid ET-18-OCH_3 has been shown to inhibit metastasis of Lewis lung carcinoma (1). Experiments in vitro with malignant mouse MO_4 cells suggested an antiinvasive activity of ET-18-OCH_3 at concentrations that permitted growth and directional migration of these cells (2). We have examined whether the antiinvasive effect of ET-18-OCH_3 on MO_4 cells was shared by other lipid derivatives, namely, a thioether-lysophospholipid, BM 41.440; an alkyl-linked lipoidal amine, CP-46,665; and a naturally occurring ester-linked phospholipid derivative, 2-LPC. We also examined whether two other malignant cell types, the LLC-H61 Lewis lung carcinoma subline (3) and the 12RIC-RK rat kidney adenovirus type 12 transfectant (4), were sensitive to the antiinvasive activity of ET-18-OCH_3. Since alterations of invasion in vitro had been ascribed to changes in cell surface glycopeptides (5), we compared the effect of ET-18-OCH_3 on invasion with that on the gel filtration profiles of cell surface fucosyl-glycopeptides from the three cell types.

MATERIALS AND METHODS

Cell lines. MO_4 cells (obtained through M. De Brabander, Janssen Pharmaceutica Research Laboratories, Beerse, Belgium) are immortalized fetal C3H mouse cells transformed by Kirsten murine sarcoma virus (6); they produced invasive and metastatic sarcomas after transplantation into syngeneic mice (7).

12R1C-RK cells (obtained through L. Smets, The Netherlands Cancer Institute, Amsterdam, The Netherlands) are baby WAG RIJ rat kidney cells, which after transfection with a genomic fragment of the oncogenic adenovirus type-12 DNA (left 0 to 16%) gave rise to tumors in nude mice (4).

LLC-H61 cells (obtained from G. Vaes, Institute of Cellular and Molecular Pathology, Brussels, Belgium) were derived from a highly metastatic subclone of a C57B1/6 mouse Lewis lung carcinoma cell line (3).

LLC-H61 cells and 12R1C-RK cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM, Flow Laboratories, Irvine, Scotland), MO_4 cells in Minimum Essential Medium Eagle (modified) with Earle's salts and nonessential amino acids (EMEM, Flow), both supplemented with 10% (v/v) fetal bovine serum, 0.05% (w/v) L-glutamine, 250 IU/ml penicillin and 100 μg/ml streptomycin (hereafter called culture medium).

Drugs. The following lipid derivatives were dissolved to concentrations between 1 and 30 μg/ml in the culture medium described with the cell lines: 2-lysophosphatidylcholine (2-LPC; F. Roth, Karlsruhe, FRG); *rac*-1-*O*-octadecyl-2-*O*-methylglycero-3-phosphocholine (ET-18-OCH_3; Medmark Chemicals, Muenchen-Gruenwald, FRG); *rac*-1-hexadecyl-mercapto-2-methoxymethyl-3-propyl-phosphoric-acid-monocholin-ester (BM 41,440; Boehringer Mannheim, Mannheim, FRG); 1-2-decycloxy-3-(4,4 amino-methyl, phenylpiperidine) (CP-46,665; Pfizer Central Research, Groton, CT). The structure of these drugs has been described (8,9).

Assay for invasion. Spheroids of MO_4 or 12R1C-RK cells (0.2 mm in diameter) or clusters of LLC-H61 cells were confronted with precultured fragments of 9-day-old embryonic chick heart (PHF) (0.4 mm in diameter) in individual cultures on a Gyrotory® shaker (10). The specificity and the relevance of this assay have been reviewed (11). Drugs were added at the onset of the culture. Confronting pairs were fixed after 4 days in Bouin Hollande's solution for embedding in paraffin and complete serial sectioning into 8-μm-thick sections. Consecutive sections were stained with hematoxylin and eosin or with an antiserum against embryonic chick heart (12). Histological analysis of invasion was based on occupation and degeneration of the PHF and scored on criteria

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Abbreviation: PHF, precultured embryonic chick heart fragment.

described earlier (13) and slightly modified: grades I and II, absence of invasion; grades II-III, a few single cells were inside the PHF without evidence of degeneration; grade III, invasion in less than half of the PHF; grade IV, invasion in more than half of the PHF.

Isolation and analysis of cell surface glycopeptides. Changes in the fucosyl-glycopeptides of the cell membrane were monitored by incubating the cells for 16 hr at 37 C in culture medium supplemented with either L-[6-^{3}H]fucose (1μCi/ml, 60 Ci/mmol) or L-[1-^{14}C]fucose (0.5 μCi/ml, 60 mCi/mmol), both purchased from the Radiochemical Center Amersham (Buckinghamshire, UK). The glycopeptides were isolated from the surface of the cell with the help of proteolytic enzymes as described (14) and dialyzed against bidistilled water. Gel filtration was performed on Bio-Gel P-10:Sephadex G-50 (2:1, w/w) columns, eluted with 0.1 M Tris-HCl buffer (pH 8.0) containing sodium dodecyl sulphate (0.1%; w/v), EDTA (0.01%, w/v) and 2-mercaptoethanol (0.1%, v/v). Cell surface glycopeptides from treated and untreated cells were investigated by cochromatography.

RESULTS AND DISCUSSION

Effect of lipid derivatives on invasion. Invasion of MO_4 cells, 12R1C-RK cells or LLC-H61 cells into PHF was

TABLE 1

Effect of Four Lipid Derivatives on the Invasion in Organ Culture of Three Types of Cells

Type of drug	Concentration of drug (µg/ml)						
	0	1	3	5	6	10	30
MO_4 cells							
2-LPC	IV(3)	n.d.	n.d.	n.d.	n.d.	IV(3)	IV(3)
ET-18OCH$_3$*	IV(3)	n.d.	n.d.	n.d.	n.d.	III(3)	II(3)
ET-18-OCH$_3$**	III(2),IV(3)	n.d.	n.d.	n.d.	n.d.	II(4),II-III(1)	n.d.
BM 41.440	IV(5)	n.d.	n.d.	IV(5)	n.d.	II(2),III(1)	II(4)
CP-46,665**	III(2),IV(3)	n.d.	IV(3)	n.d.	II-III(2)	III(1),IV(1)	n.d.
CP-46,665	III(1),IV(3)	IV(4)	IV(4)	n.d.	n.d.	IV(4)	n.d.
12R1C-RK cells							
ET-18-OCH$_3$*	III(2)	n.d.	n.d.	n.d.	n.d.	II(4)	II(3)
ET-18-OCH$_3$	III(1),IV(1)	III(2)	II(2)	n.d.	II(3)	II(3)	n.d.
LLC-H61 cells							
ET-18-OCH$_3$*	III(1),IV(2)	n.d.	n.d.	n.d.	n.d.	II(1),III(2)	III(3)

Asterisks indicate matched experiments. Roman figures are grades of invasion after 4 days, as described in Materials and Methods; number of cultures in parentheses. n.d., No data.

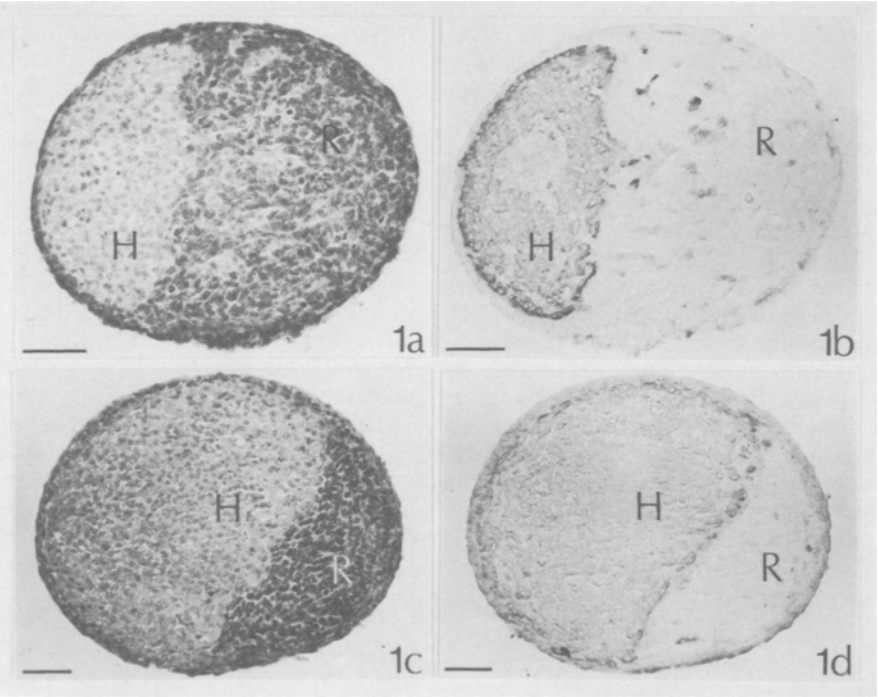

FIG. 1. Photomicrographs of sections from confronting cultures of 12R1C-RK cells (R) and embryonic chick heart (H) without (1a and 1b) and with 3 µg/ml ET-18-OCH$_3$ (1c and 1d). Fixation after 4 days; staining with hematoxylin-eosin (1a and 1c) and with antiserum against chick heart (1b and 1d). Scale bars = 50 µm.

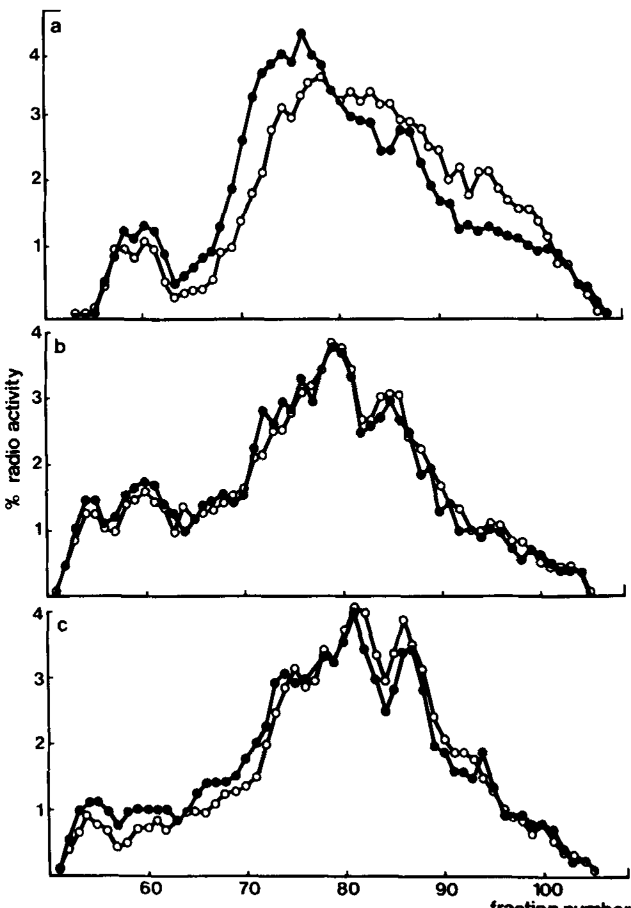

FIG. 2. Gel filtration profiles of surface glycopeptides derived from untreated (open symbols) and ET-18-OCH_3-treated (closed symbols) MO_4 (a), 12R1C-RK (b) and LLC-H61 (c) cells metabolically labeled with [^{3}H]fucose or with [^{14}H]fucose; cochromatography on Bio-Gel P-10:Sephadex G-50 columns.

obvious in all control cultures (Table 1), as described earlier (10,15). For MO_4 cells, ET-18-OCH_3 and BM 41.440 were antiinvasive in this assay; BM 41.440 had the same potency as ET-18-OCH_3, since both inhibited invasion at a concentration of 10 μg/ml. At this concentration, CP-46,665 had no effect on the invasion of MO_4 cells (Table 1). The differential antiinvasive effect of CP-46,665 vs ET-18-OCH_3 and BM 41.440 was not reflected by similar differences in their cytostatic and cytotoxic activities in vitro on cells from human tumors and leukemias (8,9). This discrepancy, together with the minor effect of ET-18-OCH_3 on MO_4 cell proliferation (2), suggested different molecular targets for antiinvasiveness as compared to cytostasis or cytotoxicity. 12R1C-RK cells (Fig. 1) were more sensitive than MO_4 cells to the antiinvasive activity of ET-18-OCH_3, whereas LLC-H61 cells were less or not sensitive (Table 1).

Effect of ET-18-OCH_3 on cell surface glycopeptides. To investigate the effect of ET-18-OCH_3 on the carbohydrate moieties of surface glycoproteins, the three types of cells were incubated for 48 hr with ET-18-OCH_3 at the lowest concentration that inhibited invasion. Figure 2 illustrates the elution profiles of glycopeptides derived from treated as compared to untreated MO_4 cells, 12R1C-RK cells and LLC-H61 cells. The glycopeptides from cells treated with ET-18-OCH_3 showed a slight shift in the gel filtration profiles toward higher apparent molecular weight classes, which was most obvious in MO_4 cells. Since the effect of ET-18-OCH_3 concerns the degree of glycopeptide sialylation (5), the differences in the effect of the drug on various types of cells might depend on the amount of sites amenable to sialylation. Figure 2 suggested that the surfaces of MO_4 as well as LLC-H61 cells contain relatively more such sites than the surface of 12R1C-RK cells.

It is unlikely that inhibition of invasion in the present experiments was due solely to alterations in glycopeptides in the malignant cells, since ranking of the sensitivity of the three cell types to the antiinvasive effect of ET-18-OCH_3 was not reflected by corresponding shifts in their glycopeptide profiles.

More probably the effects of ET-18-OCH_3 on the invasion of the three cell types have to be interpreted on the basis of glycopeptide profiles, not only from the confronting cells but also from PHF, for the following reasons: i) Nonmalignant cells transiently became invasive after pretreatment with ET-18-OCH_3, which caused similar alterations in glycopeptides as permanently present in malignant cells (5); ii) Pretreatment of PHF, causing a shift in gel filtration profiles toward that of malignant cells, inhibited invasion. One explanation of these observations is that invasion depends upon differences between cell surface glycoproteins of the PHF as compared to the confronting cells (5,16).

ACKNOWLEDGMENTS

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Ether Lipids Inhibit the Effects of Phorbol Diester Tumor Promoters[1]

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Recent studies have shown that the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) stimulates protein kinase C (PKC), whereas the ether-linked phospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol-3-phosphocholine ($ET\text{-}18\text{-}OCH_3$) inhibits PKC activity in vitro. Therefore, the antitumor effects of $ET\text{-}18\text{-}OCH_3$ could be due to its inhibition of PKC activity and the effects of tumor promotion. TPA stimulates arachidonic acid release, prostaglandin synthesis, phosphatidylcholine synthesis and the degradation of phosphatidylcholine by phospholipase C in Madin Darby canine kidney (MDCK) cells. Therefore, we have determined the effects of $ET\text{-}18\text{-}OCH_3$ on these consequences of TPA stimulation. Preliminary experiments determined that $ET\text{-}18\text{-}OCH_3$ inhibited PKC partially purified from MDCK cells by ion-exchange chromatography on DEAE-cellulose. In addition, $ET\text{-}18\text{-}OCH_3$ inhibited the TPA-stimulated phosphorylation of a 40,000-dalton protein in intact MDCK cells. These data indicate that $ET\text{-}18\text{-}OCH_3$ is an effective inhibitor of PKC activity in MDCK cells. In addition, $ET\text{-}18\text{-}OCH_3$ was found to inhibit arachidonic acid release and prostaglandin synthesis. The inhibition of prostaglandin synthesis appears to be secondary to inhibition of arachidonic acid release, since $ET\text{-}18\text{-}OCH_3$ does not inhibit TPA-stimulated synthesis of prostaglandin H synthase or the activity of the enzyme directly (Parker, J., Daniel, L. W., and Waite, M. [1987] *J. Biol. Chem. 262,* 5385–5393). $ET\text{-}18\text{-}OCH_3$ also inhibits TPA-stimulated phosphatidylcholine synthesis and phosphatidylcholine degradation by phospholipase C. These data provide evidence that the antineoplastic ether lipids inhibit the biochemical effects of the tumor promoter TPA in intact cells and indicate that this inhibition may have a role in their biological activities.

Lipids 22, 851–855 (1987).

The phorbol diester tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) causes profound changes in the metabolism of cultured cells. Among these changes are stimulated release of arachidonic acid from cellular phospholipids and increased prostaglandin synthesis (1,2).

In addition, TPA causes an increase in synthesis of choline-containing phosphoglycerides (PC) and an increase in PC degradation by a phospholipase C (Scheme 1) (3). This stimulated cycling of cellular PC results in transient increases in diglycerides. It has been suggested that this "PC cycle" provides an alternate mechanism for generating bioactive diglycerides that is independent of the turnover of phosphatidylinositol (3,4).

Thus, an inhibitor of the PC cycle would be useful in determining the biological significance of these reactions. Furthermore, these alterations in lipid metabolism are of interest since the stimulatory activity correlates well with the tumor promoting activities of the phorbol diesters (5). TPA has been shown to activate Ca^{2+}-activated phospholipid-dependent protein kinase (PKC) (6), which is thought to mediate many of the biological activities of TPA (7). Thus, it was logical to test the effects of a variety of PKC inhibitors on TPA-stimulated PC turnover. Structurally diverse inhibitors of PKC were chosen (Fig. 1): 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($ET\text{-}18\text{-}OCH_3$), 1-*S*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($ET\text{-}18S\text{-}OCH_3$), 1-*O*-octadecylpropane-

SCHEME 1. TPA-stimulated PC turnover.

FIG. 1. Structures of the PKC inhibitors.

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Abbreviations: TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; PKC, Ca^{2+}-activated, phospholipid-dependent protein kinase; MDCK, Madin Darby canine kidney; PC, choline-containing phosphoglycerides; CI, 1-(5-isoquinolinsulfonyl)piperazine; CP 46,665, 4-aminomethyl-1-[2,3-(di-n-decycloxy)n-propyl]-4-phenylpiperidine dihydrochloride; $ET\text{-}18\text{-}OCH_3$, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ET-18-H, 1-*O*-octadecylpropanediol-3-phosphocholine; $ET\text{-}18\text{-}S\text{-}OCH_3$, 1-*S*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; TLC, thin layer chromatography; PC-PLC, a TPA-stimulated phospholipase C that degrades PC.

diol-3-phosphocholine (ET-18-H) (8), 1-(5-isoquinolinsulfonyl)piperazine (CI) (9) and 4-aminomethyl-1-[2,3-(di-n-decycloxy)n-propyl]-4-phenylpiperidine dihydrochloride (CP 46,665) (10). ET-18-OCH_3 is a diether-linked phospholipid with antitumor activity (11). CI is an isoquinolinosulfonylpiperazine, structurally similar to those developed by Hidaka et al. (12); CP 46,665 is a lipoidal amine, with antineoplastic activity (13,14). Madin Darby canine kidney (MDCK) cells were used in these experiments because they had previously been shown to respond to low concentrations of TPA, and their response to TPA has been well characterized (1–3).

MATERIALS AND METHODS

MDCK cells and cell culture reagents were purchased from Flow Laboratories (Rockville, MD). $[^{32}P]_i$ (carrier-free) was obtained from ICN (Irvine, CA). [Methyl-^{3}H]-choline chloride (78 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL); En^{3}Hance and [methyl-^{3}H]choline chloride (80 Ci/mmol) were from NEN Research Products (Boston, MA). All solvents were purchased from Fisher Chemicals (Pittsburgh, PA) and were either reagent grade or redistilled in the laboratory. Cell cultures were harvested and the lipids were extracted as described by Daniel et al. (3). Phospholipids were separated by thin layer chromatography (TLC) using a solvent system of chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v). The radiolabeled products were located by autoradiography (3) or by scanning the chromatograms with a Bioscan radiochromatogram scanner (Bioscan Inc., Washington, DC). Lipid standards for TLC were obtained from Serdary Research Laboratories (London, Ontario, Canada). Silica Gel 60 plates were from E. Merck (Darmstadt, FRG). TPA, mezerein and phorbol retinoate acetate were obtained from LC Services Corp. (Woburn, MA).

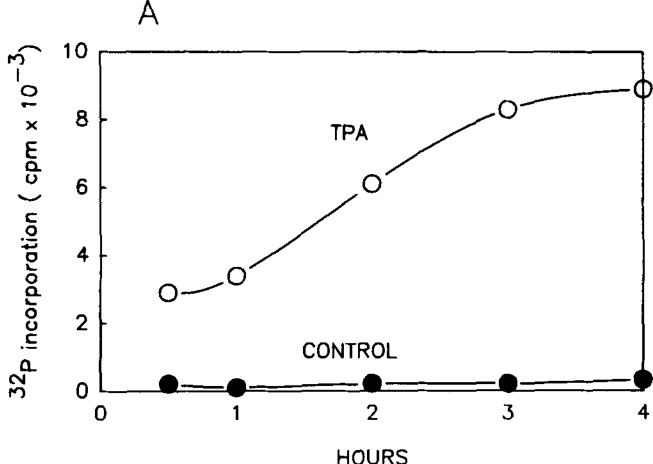
ET-18-OCH_3 and ET-18-H were gifts from Dr. W. E. Berdel, Munich, FRG. CP 46,665 was a gift from Dr. K. E. Jensen, Central Research, Pfizer, Inc., Groton, CT. CI was a gift of Dr. Craig Gerard, Bowman Gray School of Medicine, Winston-Salem, NC, and was prepared as described by Gerard et al. (9). ET-18S-OCH_3 was prepared as described previously (15).

Some variability was observed in the total incorporation of radiolabel among experiments. This variability made compiling data from different experiments difficult; therefore, the data are presented as the average of two separate samples from a representative experiment, which was one of three or more separate experiments for each graph.

RESULTS

Preliminary experiments were done to further characterize the stimulation of PC synthesis by TPA. To determine the time course of stimulation, cells were incubated in phosphate-free medium for various times with and without TPA (10 nM), and $[^{32}P]_i$ (50–100 μCi/ml) was then added for 30 min. At the end of the incubation, the medium was discarded and the incorporation of $[^{32}P]_i$ into PC was determined. There was a significant stimulation of $[^{32}P]_i$ incorporation after 30 min preincubation; however, the stimulation continued to increase with time of preincubation (Fig. 2A). In all further experiments, 3 hr was chosen for preincubation. To determine if other tumor promoters stimulated $[^{32}P]_i$ incorporation in MDCK cells, mezerein and phorbol retinoate acetate were also tested at equimolar concentrations in the range of 1 to 100 nM. These compounds and TPA were found to have similar effects on $[^{32}P]_i$ incorporation (data not shown). TPA also caused a stimulated incorporation of [^{3}H]choline into PC (data not shown).

The stimulation of $[^{32}P]_i$ incorporation was quite specific for PC; $[^{32}P]_i$ incorporation into ethanolamine-, serine- or inositol-containing phosphoglycerides was not stimulated (Fig. 2B). Our previous studies have shown that TPA does not stimulate the incorporation of $[^{32}P]_i$ into phosphatidylinositol, phosphatidylinositol 4-phosphate or phosphatidylinositol 4,5-bisphosphate (16).



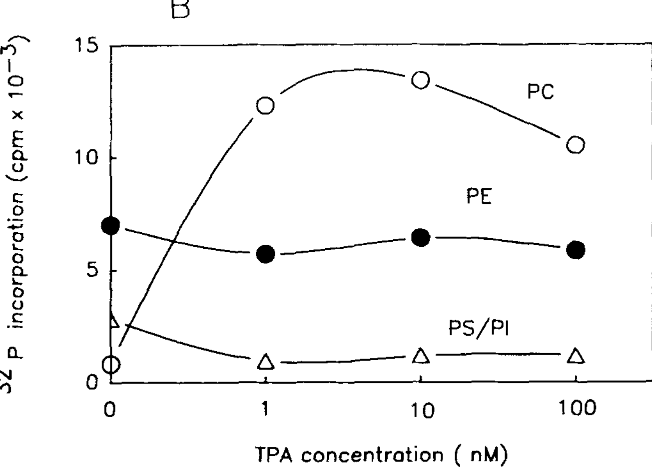


FIG. 2. Effect of preincubation time with TPA and concentration of TPA on the incorporation of $[^{32}P]_i$ into cellular phospholipids. MDCK cells (5×10^5/ml) were preincubated with TPA in serum-free, phosphate-free medium for the indicated times, and the cells were then incubated with $[^{32}P]_i$ (100 μCi/ml) for an additional 30 min. (A) ○, TPA (10 nM); ●, control without TPA. The incorporation into PC was determined as described in Materials and Methods. Cells were also incubated with varying concentrations of TPA for 3 hr and then with $[^{32}P]_i$ as described above, and the incorporation into cellular phospholipids was determined as described in Materials and Methods. (B) ○, PC; ●, phosphatidylethanolamine; △, phosphatidylserine plus phosphatidylinositol.

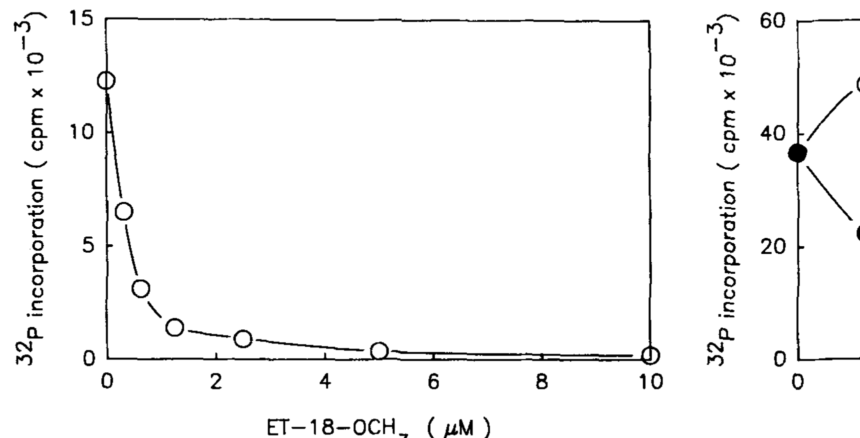


FIG. 3. Effect of ET-18-OCH$_3$ on TPA-stimulated PC synthesis. MDCK cells (5×10^5/ml) were incubated with TPA (10 nM) and the indicated concentration of ET-18-OCH$_3$ for 3 hr, and [^{32}P]$_i$ was added for an additional 30 min.

FIG. 4. Effect of ET-18-H and CP 46,665 on TPA-stimulated PC synthesis. ET-18-H (●) and CP 46,665 (○) were tested using conditions identical to those described in the legends to the previous figures.

FIG. 5. Effect of ether lipid analogs on TPA-stimulated PC hydrolysis. MDCK cells (5×10^5/ml) were prelabeled for 18 hr with [^{3}H]choline (1 µCi/ml); then the medium was removed and replaced with fresh medium containing 10^{-7} M TPA (○) or without TPA (●), and the concentrations of inhibitors were indicated. The release of radiolabel into the medium was determined after 1, 2 and 4 hr of incubation. The data presented represents 4 hr of incubation.

The simultaneous addition of ET-18-OCH_3 during the preincubation of MDCK cells caused a marked dose-dependent suppression of the stimulation of $[^{32}P]_i$ incorporation into PC (Fig. 3). ET-18S-OCH_3 was equally effective when tested under identical conditions (data not shown). When CI was tested in three separate experiments under conditions identical to those given above, no suppression of $[^{32}P]_i$ incorporation into PC was observed in the concentration range of 5 to 500 μM (data not shown). Similarily, CP 46,665 was tested in the concentration range of 1 to 10 μM, and no inhibition of TPA-stimulated $[^{32}P]_i$ incorporation into PC could be demonstrated (Fig. 4). The alkylphospholipid ET-18-H, which is structurally similar to ET-18-OCH_3, was tested and found to inhibit PC synthesis (Fig. 4). The concentration dependence of ET-18-H is similar to ET-18-OCH_3; however, ET-18-H is somewhat less effective.

We have previously shown that the release of water-soluble radioactive compounds from cells prelabeled with [^{3}H]choline is due to a TPA-stimulated phospholipase C that degrades PC (PC-PLC) (3). Therefore, quantitation of water-soluble radiolabel was used to test the effects of PKC inhibitors on PC-PLC. Phospholipids were also extracted and identified by TLC (3) to confirm that PC was the source of the water-soluble product (data not shown). Cells were prelabeled with [^{3}H]choline for 18 hr, then treated with varying concentrations of PKC inhibitors, with or without TPA (10^{-7} M). In these experiments, [^{3}H]choline release was greater in TPA-stimulated cells than in control cells (Fig. 5). ET-18-OCH_3 (Fig. 5A) and ET-18S-OCH_3 (Fig. 5B) inhibited the release of [^{3}H]choline. However, two other PKC inhibitors CI (Fig. 5C) and CP 46,665 (Fig. 5D) were without effect.

DISCUSSION

Vogler et al. (17) recently demonstrated that ET-18-OCH_3 inhibits [^{3}H]choline incorporation into HL60 cells. These authors did not use TPA-stimulated cells; however, they suggested that the mechanism by which ET-18-OCH_3 inhibits PC synthesis may be due to protein kinase C inhibition. The data reported here suggest that the inhibition of PC synthesis by ET-18-OCH_3 and ET-18-H does not involve protein kinase C, since two other inhibitors of protein kinase C, CI and CP 46,665, when used at concentrations proven effective in preventing other effects of TPA treatment, are without effect. Our related studies (18) have shown that ET-18-OCH_3 and CI inhibit PKC partially purified from MDCK cells by DEAE-cellulose chromatography. In addition, both compounds are effective at equimolar amounts in inhibiting TPA-induced arachidonic acid release and prostaglandin synthesis in MDCK cells. This is noteworthy, since CI was without effect in the present study when used at 400-fold molar excess of the IC_{50} for ET-18-OCH_3. The concentrations of CI tested were greater than that required to inhibit TPA or diglyceride-stimulated respiratory burst in human neutrophils (7). Previous studies with CP 46,665 have shown that it is as effective as ET-18-OCH_3. For example, CP 46,665 is as effective as ET-18-OCH_3 in an assay to measure protein kinase C inhibition (8) and in assays to measure cytostatic activity (11).

ET-18-OCH_3, ET-18S-OCH_3 and ET-18-H are structurally similar to lyso-PC, which has been shown to be a potent inhibitor of CTP:phosphocholine cytidylyltransferase, the rate-controlling enzyme in PC synthesis (19), and CDP choline:1,2-diacylglycerol choline phosphotransferase, the last enzyme in PC synthesis (20). Therefore, the alterations in PC metabolism by ET-18-OCH_3, ET-18S-OCH_3 and ET-18-H may be due to their structural similarity to lyso-PC rather than to their effects on protein kinase C. The structural similarities of these ether lipids to lyso-PC may also be important with regard to their inhibition of lyso-PC acyltransferase (21), the inhibition of which correlates with cellular cytotoxicity (22).

These data suggest that protein kinase C is not involved in TPA-stimulated PC synthesis and that TPA may have biological effects other than those involving protein kinase C. This view is supported by recent reports that synthetic diacylglycerides (23,24) and bryostatin (24) stimulate protein kinase C in HL60 cells, but unlike TPA do not induce differentiation (26) or changes in PC metabolism (23).

Diacylglycerol is a precursor of PC and when added exogenously could stimulate PC synthesis by providing more substrate for the CDP choline:1,2-diacylglycerol cholinephosphotransferase. However, exogenous glycerides are converted primarily to the corresponding phosphatidic acid derivative by HL60 cells (27) and do not mimic the TPA-induced changes in PC metabolism. Recent studies have demonstrated that the TPA-induced alteration in PC metabolism results from a cyclic breakdown and resynthesis of PC (3). This pathway is similar to the phosphatidylinositol cycle and provides an alternate source of diglycerides. The significance of this "PC cycle" remains to be determined. Therefore, the lipid inhibitors of this cycle that are structural analogs of lysophospholipids may be useful tools in determining the role of altered PC metabolism in the mechanism of tumor promotion.

ACKNOWLEDGMENTS

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Metabolism of Ether Phospholipids and Analogs in Neoplastic Cells[1]

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The ether phospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (OM-GPC) is known to be a potent inhibitor of cell growth. Metabolic studies in both Raji and L1210 leukemic cells on OM-GPC, ^{3}H-labeled in the methyl groups of the choline moiety, showed a (diacyl)-phosphatidylcholine as the only labeled metabolite. Since the formation of radiolabeled (diacyl)-phosphatidylcholine showed a direct correlation with cell death, we tested other lipid analogs. One of these compounds, hexadecylphosphocholine (He-PC), which was ^{3}H-labeled in the methyl-choline groups, showed a formation of labeled (diacyl)-phosphatidylcholine similar to that found with OM-GPC. Again, there was a direct linear correlation between the formation of the labeled product and cell death. He-PC was found to be a potent cell toxin in in vitro experiments on cell cultures. However, analogs with an elongated phosphor to trimethylammonium distance showed no toxicity towards the cells in in vitro experiments. From the data, we conclude that the ether phospholipids are substrates for a phospholipase C or related enzyme. This subtrate property may be responsible for the toxicity of the compounds in neoplastic cells.
***Lipids 22,* 856-861 (1987).**

The (ether)-phospholipid 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (OM-GPC) is known to be a potent inhibitor of cell growth (1-7). It is known to stimulate macrophages (8), destroy tumor cells in vitro, and is suggested to be more lethal to tumor cells than to normal cells. The hypothesis on the selectivity of the compound towards neoplastic cells is based on the observation of Soodsma et al. (9) that there is a difference in the level of alkyl glyceromonooxygenase (AGMO; E.C.1.14.16.5) between normal cells and several neoplastic cells. However, later studies showed that OM-GPC is not a substrate for AGMO in vitro (10-12). Thus, the selective action of OM-GPC to tumor cells cannot be explained by the cellular levels of AGMO. This view also is supported by a recent study in which AGMO levels do not show any correlation with the toxic effects of OM-GPC in different cell lines (13). To understand its metabolic fate and learn about its mechanism of action, we synthesized OM-GPC, ^{3}H-labeled in the methyl groups of the choline moiety. The metabolism of the radiolabeled compound in Raji and L1210 leukemic cells was investigated. Analysis of the results on the metabolic fate of these molecules in the different cell lines enabled us to design a new class of antineoplastic agents, the alkyl phosphocholines.

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Abbreviations: OM-GPC, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-hoshocholine; OM-G, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol; He-PC, hexadecylphosphocholine; Ol-PC, octadecenyl(9,10)phosphocholine; Ol-P(C_6)C, octadecenyl(9,10)phospho-(N,N,N-trimethylamino)hexanol.

MATERIALS AND METHODS

1-*O*-Octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, hexadecylphosphocholine, octadecenyl(9,10)phosphocholine and octadecenyl(9,10)phospho-(N,N,N-trimethylamino)hexanol were prepared essentially as described elsewhere (14-16). Radiolabeled 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phospho[^{3}H-methyl]choline and hexadecylphospho[^{3}H-methyl]choline were prepared in our laboratory (17), using [^{3}H]methyliodide (Amersham, Braunschweig, FRG). Other chemicals were of analytical grade and used without further purification.

Raji cells (B-lymphoblastic lymphome) and L1210 cells (murine leukemic cells) were grown in RPMI 1640 medium (GIBCO, Karlsruhe, FRG), supplemented with 10% fetal calf serum (Biochrom, Berlin, FRG). Streptomycin (200 mg/l), nystatin (2.10^4 IE/l), and penicillin (2.10^5 IE/l) were added routinely to the culture medium.

Incubation of cells (10 ml, 2×10^5 cells/ml) was performed with 2.5 μg/ml OM-GPC or 10 μg/ml of alkyl phosphocholine per ml. After the indicated time periods, aliquots of the cell suspension were counted in a Neubauer cell-counting chamber and tested for viability using the trypan blue dye exclusion test (18). The cell suspension was then diluted with 10 ml serum-free medium, and the cells were centrifuged at 800 g for 10 min at room temperature. The supernatant was collected, and the cells were washed twice with 10 ml serum-free medium. Of the combined supernatants (40 ml), 100 μl aliquots were taken for ^{3}H counting. The pellet was extracted twice according to Bligh and Dyer (19). The extracts were combined; 2.5 ml water and 2.5 ml chloroform were added for phase separation. The chloroform layer (lower phase) was collected, and the water phase was reextracted with 5 ml chloroform. After phase separation, aliquots of the water and chloroform phases were taken for ^{3}H counting. The chloroform phase was dried under a stream of nitrogen and redissolved in a small volume of chloroform/methanol (9:1, v/v). The organic material was applied to a thin layer chromatography plate, and the plate was developed in chloroform/methanol/acetic acid/water 100:60:20:5, v/v/v/v). After drying, the plate was scanned for radioactive products in an Automatic Linear Analyzer LB 284 complemented with a Data System LB 500 (Berthold, Wildbad, FRG).

Uptake of OM-GPC by the cells was determined by incubation with radiolabeled compound (S.A. 200 mCi/mmol). The cells (2×10^6 cells in 2 ml medium containing 20 μg OM-GPC) were incubated for the indicated time period and then immediately diluted with 4 ml ice-cold medium containing 20% (v/v) fetal calf serum. The cells were centrifuged 10 min at 800 g, and the supernatant was discarded. The cells were washed twice with 10 ml ice-cold medium containing 10% fetal calf serum. After the last centrifugation, the pellet was submitted to a Bligh and Dyer extraction (19). Aliquots of the organic phase were used to determine the amount of radiolabel taken up by the cells.

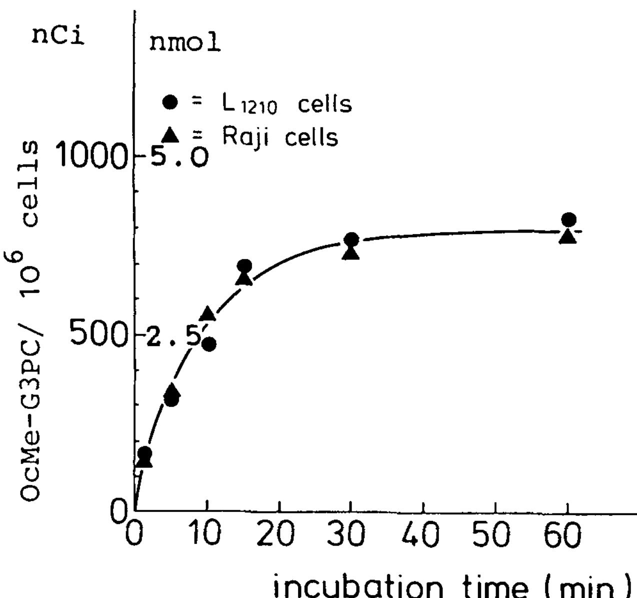

FIG. 1. Kinetics of uptake of OM-GPC in Raji and L1210 cells. Cells were incubated in a RPMI-medium containing 2.5 μg/ml of radiolabeled compound (200 mCi/mmol). At indicated time intervals, the cells were harvested directly after dilution of the cell suspension with an ice-cold RPMI-medium that contained 20% fetal calf serum. After being washed twice with the medium, the cell pellet was extracted according to Bligh and Dyer (19), and the organic phase was counted for radioactivity.

Methanolysis. The newly formed metabolite with Rf-values similar to lecithin was eluted from the thin layer plate with methanol. About 50 nmol lecithin containing 100 nCi ^3H label was treated with a solution of 200 mg potassium tert butylate in 2.5 ml methanol. After 15 min incubation at 50 C, 2 ml each of NaCl saturated water and chloroform were added. The mixture was vigorously shaken. After phase separation aliquots of both phases were counted for radioactivity.

RESULTS AND DISCUSSION

Raji and L1210 cells were incubated with the radiolabeled (ether)-phospholipid OM-GPC. As shown in Figure 1, the rate of uptake was similar for both cell lines; equal amounts of OM-GPC were taken up by both cell lines. From the data, it is evident that the cells already show maximal uptake of the compound after 60 min. Beyond this period, the concentration of OM-GPC per 10^6 cells remains constant (800 pmol/10^6 cells). Additional uptake depends only on the growth rate of the respective cell line.

During the incubation period of three days, we observed the following time dependent effects (Fig. 2). In the case of Raji cells, there is almost no effect on cell growth during the first 48 hr, whereas L1210 cells showed a markedly reduced growth rate. At the end of the third day, cell growth is almost completely stopped for both cell lines. As shown in Figure 3, similar results were obtained using the trypan blue dye exclusion test. The number of trypan

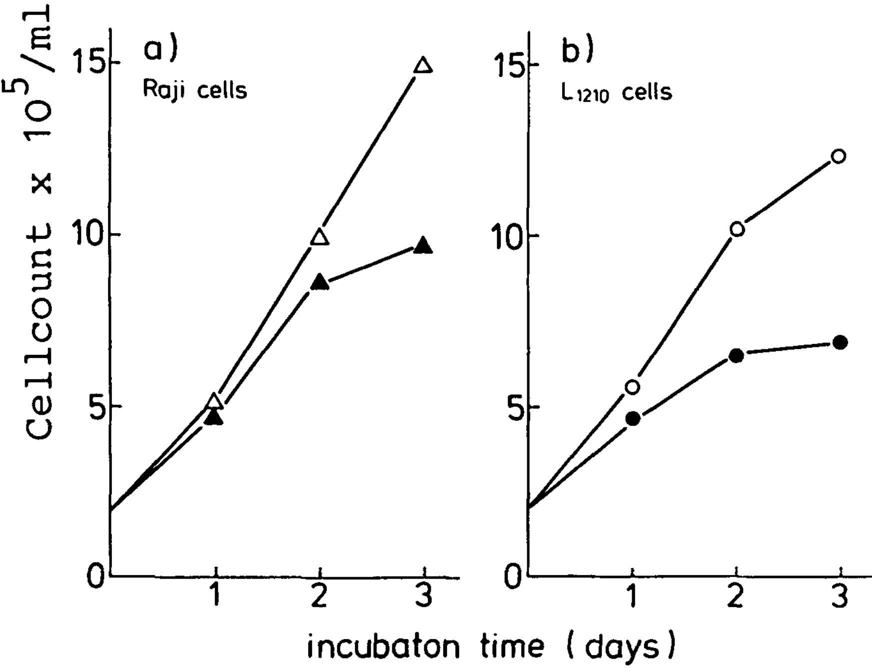

FIG. 2. Effect on cell growth of 2.5 μg OM-GPC/ml for Raji (a, n = 4) and L1210 (b, n = 5) cells. Cells were grown in a RPMI-medium containing 10% fetal calf serum. Cells were counted in a Neubauer cell-counting chamber. Open symbols: control cells (without OM-GPC). Closed symbols: cells in contact with OM-GPC. Standard deviations were 5–10% of total cell count.

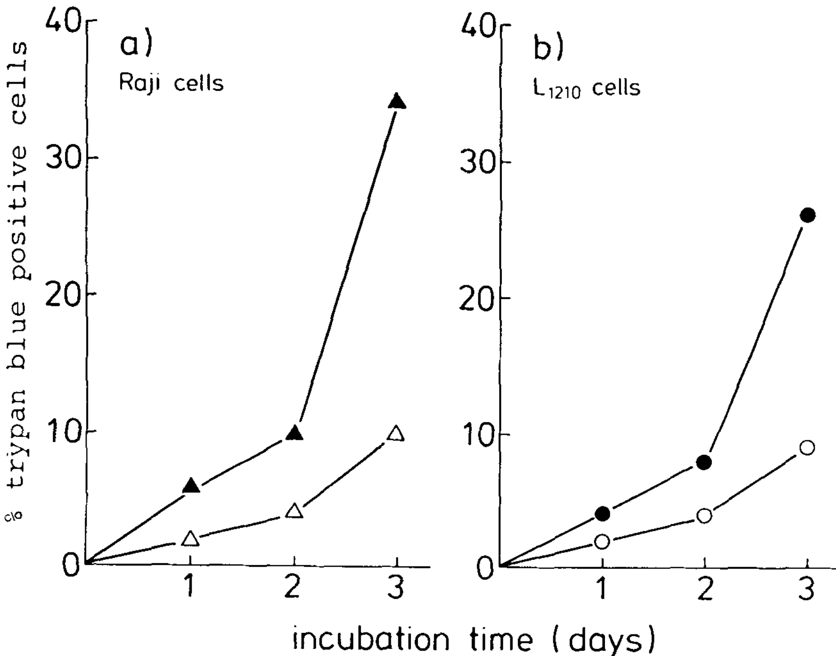

FIG. 3. Effect of OM-GPC on the cell viability for (a) Raji cells and (b) L1210 cells, measured by the trypan blue dye exclusion assay (18). Incubation conditions are as in Fig. 2. Open symbols: cells without OM-GPC. Closed symbols: cells in contact with OM-GPC. Viability data are ±5% (of total cell count) standard deviation.

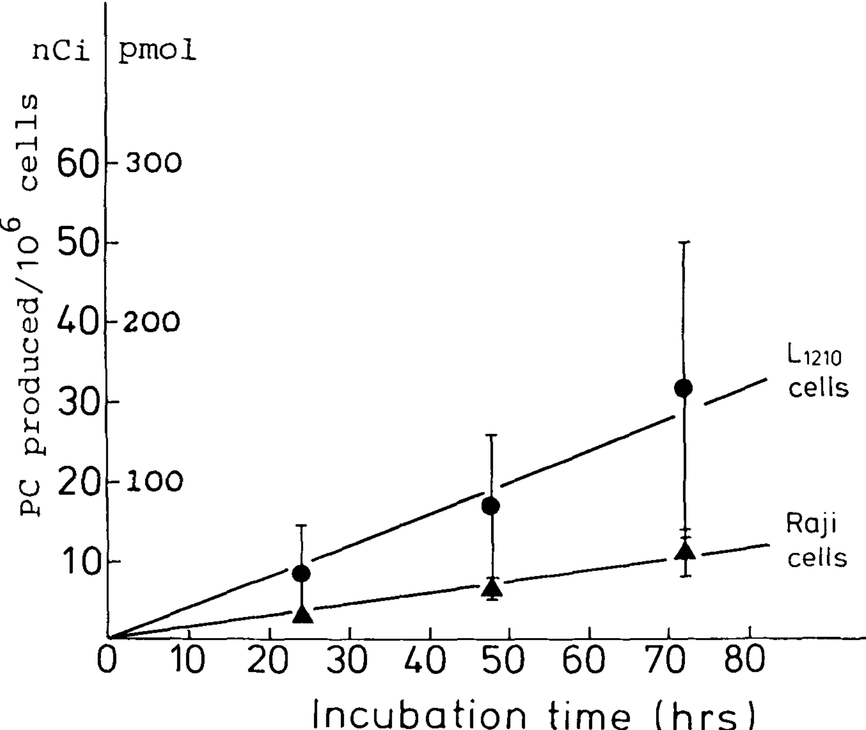

FIG. 4. Time-dependent formation of radiolabeled phosphatidylcholine in in vitro cultures of Raji and L1210 cells. Incubation conditions are as in Fig. 2. Error bars indicate standard deviations.

blue positive cells was low for the first 48 hr (<10%) but much higher after 72 hr (>25%).

The lipid extracts prepared according to Bligh and Dyer (19) were analyzed for radioactivity both in the water and in the chloroform phase. No radiolabeled material was found in the water phase. All of the label taken up by the cells (up to 12% of the label added, 800 pmol per 10^6 cells) was in the organic phase. Thin layer chromatography of the organic phase showed that besides the original compound, only one other radiolabeled product, which had a Rf-value equal to that of phosphatidylcholine, was present. As shown in Figure 4, the amount of product formed per 10^6 cells showed a linear increase with time. After three days, about 10% of the radioactivity taken up by the cells is incorporated in the lecithin fraction. After isolation of the radiolabeled product and treatment with potassium *t.* butylate, the label is detected in the water phase and not in the organic phase, indicating that the apolar part of the product is not originating from OM-GPC. The amount of phosphatidylcholine formed per 10^6 cells and the percentage of trypan blue positive cells show a direct linear correlation (Fig. 5) for both Raji cells and L1210 cells. Compared to L1210 cells, Raji cells obviously show a higher percentage of trypan blue positive cells at the same point of lecithin formation.

The metabolic fate of OM-GPC in both cell lines suggests that OM-GPC could be a substrate for phospholipase C or phospholipase C–like enzymes. According to this hypothesis, compounds of simpler molecular structure, such as the alkyl phosphocholines, should have similar effects on the cells. Indeed, as is shown in Figure 6, Raji cells treated with hexadecylphosphocholine (He-PC) do show production of lecithin. Again, the amount of trypan blue positive cells shows a linear correlation with the amount of lecithin formed from HE-PC as a phosphocholine donor.

Related compounds differing only in the distance between the phosphate and trimethylammonium groups further support this hypothesis. For instance, alkyl phosphocholines such as octadecenyl(9,10)phosphocholine (Ol-PC) and octadecenyl(9,10)phospho-(N,N,N-trimethyl)-aminohexanol (Ol-P(C6)C) behave as predicted (Figs. 7 and 8). Ol-PC is a substrate for phospholipase C and is toxic for Raji cells. Ol-P(C6)C is not a substrate for phospholipase C because of its elongated phosphor to nitrogen distance (20,21), and therefore it does not inhibit the growth of Raji cells.

The transfer of radiolabeled phosphocholine from an ether phospholipid to another molecule, forming a radiolabeled lecithin as described in this study, has not been described before. However, Voelker and Kennedy (22) reported a similar reaction, i.e. the transfer of phosphocholine from phosphatidylcholine to ceramide, forming sphingomyelin and diacylglycerol. In analogy, we suggest that a similar transfer of phosphocholine from an ether phospholipid to diacylglycerol occurs in Raji cells. The result is the formation of lecithin and the appearance of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerol, a product that was detected by Van Blitterswijk et al. (25) in similar experiments. These authors report that this metabolite inhibits protein kinase C. Their observation that OM-G is a product from OM-GPC also supports our suggestion of a transfer of phosphocholine. The reaction products of this transfer would be labeled phosphatidylcholine and OM-G or a fatty alcohol, depending on the compound used. Since the lecithin formed is a normal membrane constituent, we suspect that the toxic principle in the metabolism of alkyl phosphocholines is the formation of these apolar alcohols.

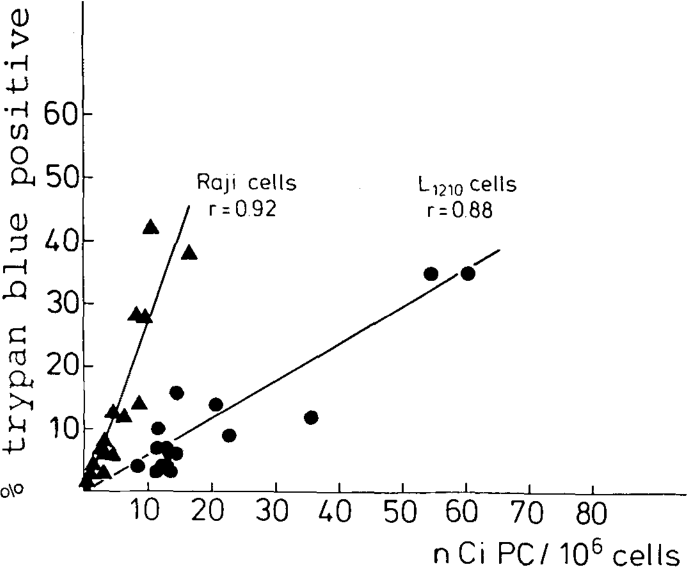

FIG. 5. Correlation of formation of phosphatidylcholine from OM-GPC and cell death (as measured by trypan blue positive cells). Each data point represents one cell culture. Incubation conditions as described in Fig. 2.

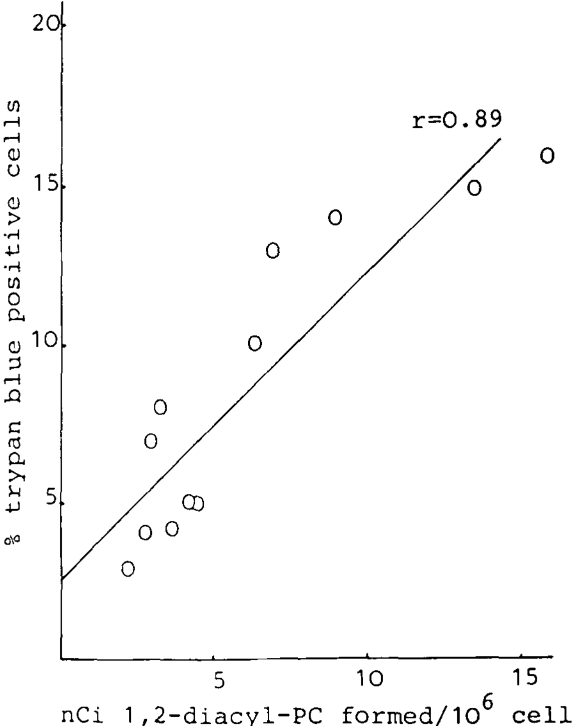

FIG. 6. Correlation between cell death (trypan blue positive cells) and phosphatidylcholine formation from hexadecylphosphocholine for Raji cells. Incubation essentially as described in Fig. 2, except that OM-GPC was replaced by 10 μg/ml of hexadecylphosphocholine. Each data point represents one cell culture.

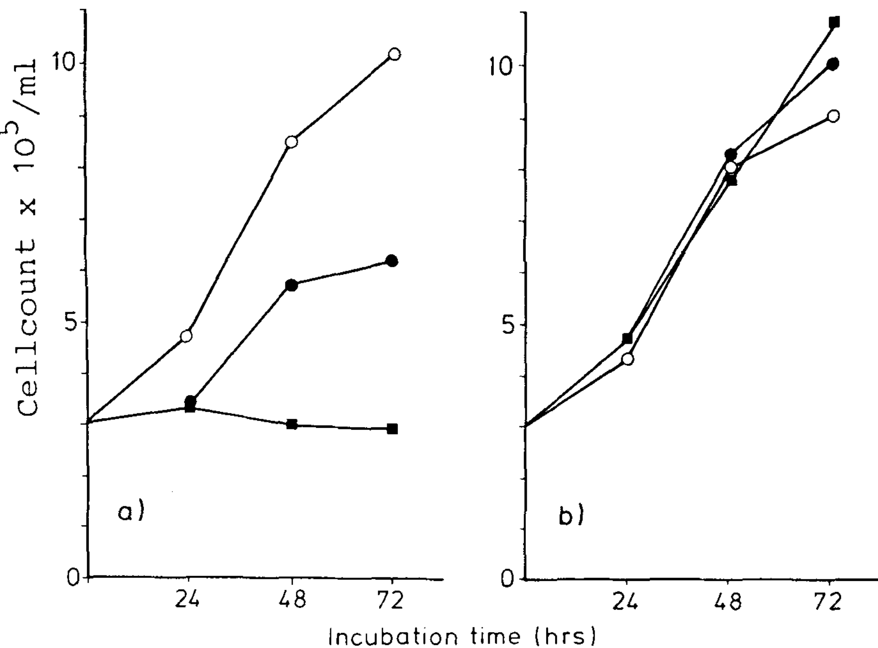

FIG. 7. Influence of elongation of the P-N distance in the alkyl phosphocholines on proliferating the Raji cells. Incubation conditions are described in Fig. 2. OM-GPC was replaced by the respective alkyl phosphocholine. Standard deviations are 5–10% of the total cell counts. Open symbols: cells without alkyl phosphocholines. Closed circles: 10 μg/ml alkyl phosphocholines. Closed squares: 40 μg/ml alkyl phosphocholines. (a) Octadecenyl(9,10)phosphocholine; (b) octadecenyl(9,10)phospho-(N,N,N-trimethylamino)hexanol.

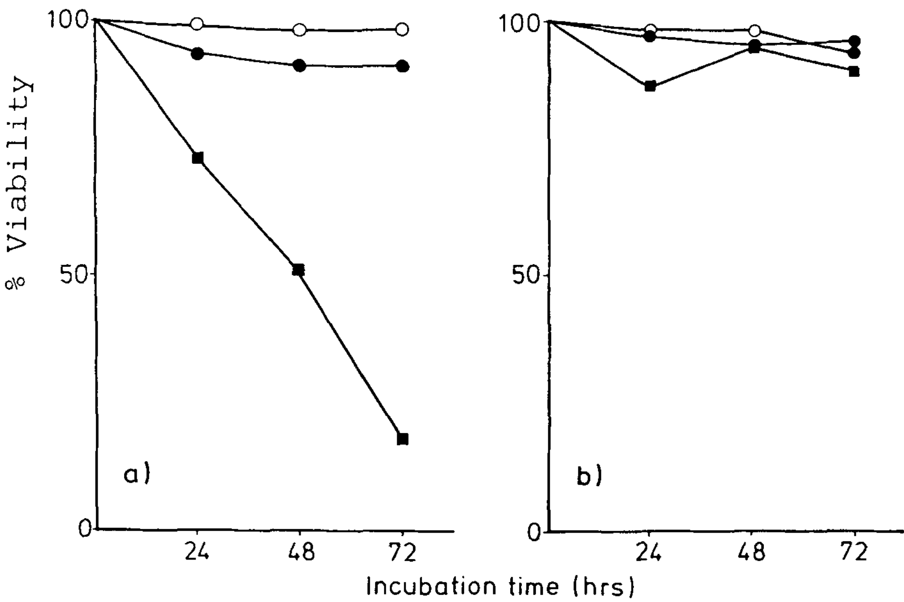

FIG. 8. Influence of elongation of the P-N distance in alkyl phosphocholines on cell viability in Raji cells measured by the trypan blue dye exclusion assay. Viability data are ±5% (of total cell count) standard deviation. Incubation conditions as described in Figs. 2 and 7. Symbols as in Fig. 7. (a) Octadecenyl(9,10)phosphocholine; (b) octadecenyl(9,10)phospho-(N,N,N-trimethyl)aminohexanol.

In summary, the formation of a metabolic product from either OM-GPC or alkyl phosphocholine shows a linear correlation with cell death as measured by the trypan blue dye exclusion assay. Related compounds, which are not a substrate for phospholipase C, do not show inhibition of growth for Raji cells. It is suggested that the formation of apolar alcohols by phospholipase C or related enzymes could be responsible for the toxicity of the ether phospholipids (23,24). The mode of action of these alcohols is not understood yet but their capacity to inhibit protein

kinase C (23) could play a role in their toxicity towards neoplastic cells.

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Antitumor Activity of Synthetic Alkylphospholipids With or Without PAF Activity[1]

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1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OMe) has been reported to possess definite antitumor activity in vivo. Twenty-two alkyl lysophospholipid analogs were chemically synthesized, and their antitumor activity against mouse experimental tumors (Sarcoma 180, MM46, P388) was examined. Among them, 1-*O*-octadecyl-2-*O*-acetoacetyl-*rac*-glycero-3-phosphocholine was found to show antitumor activity similar to ET-18-OMe with less acute toxicity.

Intravenous injection of the ET-18-OMe with *sn*-3 configuration retarded the subcutaneous growth of Sarcoma 180 cells effectively, while the growth inhibition by the *sn*-1 isomer was much less effective. This stereospecificity was similar to that observed in their activities as platelet-activating factor (PAF) agonists. The acetoacetyl compound, another PAF agonist, showed similar stereospecific antitumor action in vivo. These findings suggest that some alkyl lysophospholipids may activate host cells to a cytostatic stage against tumor cells in vivo through binding to a PAF receptor. Our preliminary results indicated that the responsible cells under these conditions might be primarily immature macrophages present in the bone marrow. No appreciable or even adverse stereospecificity was observed in the different sets of experiments where the activity of ET-18-OMe against MM46 tumor cells in vivo or the direct cytotoxicity against human promyelocytic leukemia HL-60 cells in vitro was examined. Under some conditions, the antitumor activity of ET-18-OMe in vivo may be revealed through direct cytotoxicity and/or modulation of the host defense system by "nonspecific" mechanisms. Some alkylphospholipids without PAF activity may also show antitumor activity through similar "nonspecific" mechanisms.

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Some alkyl lysophospholipids (ALP), such as 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (ET-18-OMe), have been known to possess definite antitumor effects in vivo (1). The mode of action was partially explained by selective cytotoxic action against malignant cells (2,3). The specificity originally was explained by the lack of the degradation (*O*-alkyl cleavage) enzyme in the tumor cells; however, this has recently been challenged (4). Alternative explanations for the mechanism of cytotoxic activity and its tumor specificity have been advanced (5,6), but further work is required to clarify this interesting characteristic.

In addition to the direct action, Berdel et al. proposed the possible participation of macrophages activated by ALP in suppression of tumor cell growth. For instance, they showed that intravenous injections of bone marrow macrophages preincubated with ET-18-OMe inhibited the development of metastasis of 3-Lewis lung carcinoma in C57B1/6 mice (7). No information, however, has been available about the mechanism of macrophage activation. The unique phospholipid, PAF, first characterized as a mediator released from IgE-sensitized rabbit basophils (8), has been suggested to act as a potent mediator in pathological processes such as anaphylaxis and inflammation (9). We have recently shown that macrophages as well as platelets and neutrophils are target cells of PAF through specific receptors present on their cell surfaces (10).

In the present study, we examined the relationship between the structure of ALP analogs and their antitumor activity. Our results suggest that the antitumor activity of some ALP might be at least partially through induction of activation of bone marrow macrophages in vivo and that the activation is through their binding to PAF receptors on these cells.

MATERIALS AND METHODS

Chemicals. RPMI1640 and D-MEM media were obtained from Flow Laboratories (Stanmore, Australia). Fetal calf serum (FCS) was purchased from Gibco (New York, New York). [^{3}H]Thymidine was purchased from the Radiochemical Centre (Amersham, United Kingdom). Sheep red blood cells were obtained from Nippon Bio-Supply Center (Tokyo, Japan).

Analogs of alkylphospholipids. All of the alkylphospholipids bearing O-methyl residues at position 2 (10,11), 1-*O*-octadecyl-2-*O*-acetoacetyl-glycero-3-phosphocholine (acetoacetyl compound) (12), 1,2-di-*O*-alkyl-glycerophosphocholine (1,2-dialkyl-GPC, α type) (11,13,14) and 1,3-di-*O*-dialkyl-GPC (β type) (15) used in the present study were synthesized by investigators at Takeda Chemical Industry Ltd. They were purified by column chromatography followed by crystallization from a suitable solvent system, and their purities and structures were confirmed by thin layer chromatography, nuclear magnetic resonance and elemental analysis.

Animals and antitumor test. Specific pathogen-free female ICR and C3H/HeN mice (6–8 wk old) were purchased from the Shizuoka Cooperative for Experimental Animals (Hamamatsu, Japan). The tumor cells used were Sarcoma 180 (S180) obtained from the Institute of Microbial Chemistry (Tokyo, Japan) and mouse mammary tumor MM46, from Teikyo University (Kanagawa, Japan). They were maintained in the ascites form. The 7-day-old S180 ascites tumor cells were transplanted intraperitoneally (1×10^5) or subcutaneously (1×10^6) into ICR mice. Test samples were administered intraperitoneally or

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intravenously. The 7-day-old MM46 ascites tumor cells (1×10^4) were transplanted intraperitoneally into C3H/HeN mice, with the test sample also being administered intraperitoneally.

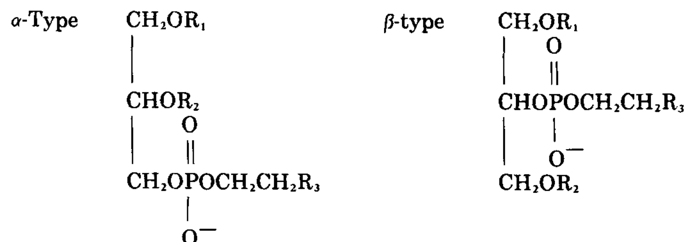
P388 mouse leukemia cells from the Cancer Institute (Tokyo, Japan) and human promyelocytic leukemia HL-60 cells from the Jichi Medical School (Tochigi, Japan) were maintained in vitro and used for cytotoxic assay.

Assay for cytostatic activity. The cell-mediated cytostasis assay was performed as described previously (16). Briefly, test samples were administered intraperitoneally four days prior to sacrifice. Peritoneal cells were harvested and coincubated with EL-4 mouse leukemia cells from the National Institute of Health, Tokyo, Japan. The radioactivity of [^{3}H]thymidine incorporated into acid-insoluble materials was then measured.

Assay for plaque-forming cells. Direct plaque-forming cell assay was performed by the method of Jerne and Nordin (17). Sheep red blood cells (5×10^7) were injected intravenously into ICR mice 13 days after subcutaneous

TABLE 1

Antitumor Activity of Synthetic Alkyl Lysophospholipids



Compound	R_1	R_2	R_3	S180 (T/C[e],S/T[f]) A[a]	S180 B[b]	S180 C[c]	MM46 (T/C,S/T) D[d]	P388[g] (IC_{50}, μM)
α-Type	$C_{12}H_{25}$	CH_3	$N^+(CH_3)_3$		124, 0/5			
	$C_{13}H_{27}$	CH_3	$N^+(CH_3)_3$		116, 0/5			
	$C_{14}H_{29}$	CH_3	$N^+(CH_3)_3$		139, 0,5			
	$C_{15}H_{31}$	CH_3	$N^+(CH_3)_3$		188, 1/5			
	$C_{16}H_{33}$	CH_3	$N^+(CH_3)_3$		252, 0/5		92, 2/3	
	$C_{17}H_{35}$	CH_3	$N^+(CH_3)_3$		213, 0/5		107, 2/5	
ET18-OMe	$C_{18}H_{37}$	CH_3	$N^+(CH_3)_3$		169, 1/5	235, 0/5	214, 3/5	4.0
	$C_{19}H_{39}$	CH_3	$N^+(CH_3)_3$		144, 1/5		105, 2/4	
	$C_{22}H_{45}$	CH_3	$N^+(CH_3)_3$	142, 0/5	207, 1/5		83, 0/4	
	$C_{18}H_{37}$	CH_3	$N(CH_3)_2$	180, 0/5	195, 0/5		113, 3/5	
	$C_{18}H_{37}$	CH_3	$NHCH_3$	175, 0/5			115, 1/5	
	$C_{18}H_{37}$	CH_3	NH_2	115, 0/5			98, 0/5	
Acetoacetyl compound	$C_{18}H_{37}$	$C(=O)CH_2C(=O)CH_3$	$N^+(CH_3)_3$	200, 0/5		238, 1/5	263, 3/5	34
	C_6H_{13}	C_6H_{13}	$N^+(CH_3)_3$	88, 0/1				
Dioctyl-GPC	C_8H_{17}	C_8H_{17}	$N^+(CH_3)_3$	257, 2/5		275, 0/5	99, 3/5	145
	C_9H_{19}	C_9H_{19}	$N^+(CH_3)_3$	184, 1/5				52
	$C_{10}H_{21}$	$C_{10}H_{21}$	$N^+(CH_3)_3$	178, 0/5		145, 0/5	110, 0/5	41
	$C_{12}H_{25}$	$C_{12}H_{25}$	$N^+(CH_3)_3$	114, 0/5				56
	$C_{16}H_{33}$	$C_{16}H_{33}$	$N^+(CH_3)_3$	117, 0/5				>300
β-Type	C_8H_{17}	C_8H_{17}	$N^+(CH_3)_3$	245, 1/5		274, 1/5	112, 1/5	100
	C_9H_{19}	C_9H_{19}	$N^+(CH_3)_3$			163, 1/4	112, 0/5	
	$C_{10}H_{21}$	$C_{10}H_{21}$	$N^+(CH_3)_3$	221, 0/5		139, 0/5	114, 1/4	

[a,b,c]ICR mouse was transplanted intraperitoneally with S180 tumor (1×10^5 cells).

[a]A test sample (1 mg per mouse per day) was administered intraperitoneally once 4 days prior to tumor transplantation.

[b]A test sample (0.25 mg per mouse per day) was administered intraperitoneally once a day on days −5, −4, −3 and −2.

[c]A test sample (0.3 mg per mouse per day) was administered intraperitoneally once a day on days 0, 1 and 2.

[d]C3H/HeN mouse was transplanted intraperitoneally with MM46 tumor (1×10^4 cells). A test sample (0.25 mg per mouse per day) was administered intraperitoneally once a day on days −5, −4, −3, −2, 2, 3, 4 and 5.

[e]Percent average life span of tested mice/control mice.

[f]Survivors of tested mice/total mice.

[g]Mouse leukemic cell P388 was cultured in a medium (RPMI1640 with 10% FCS) containing various concentrations of lipids. The viability of treated cells was examined after 24 hr by measuring ^{3}H thymidine incorporation into acid-insoluble fraction. Cultures were pulsed with ^{3}H thymidine 3 hr before termination of incubation.

inoculation of S180 tumor cells (1×10^6). Five days after immunization, splenic cells were prepared and assayed for plaque-forming cells.

RESULTS

Antitumor activity of ALP analogs. Twenty-two ALP analogs were snythesized, and their structure-antitumor activity relationships were examined (Table 1). Those included analogs differed from ET-18-OMe in the structure of the long chain alkyl moiety at position 1 or the N-substituted aminoethylphosphoryl moiety at position 3. Prophylactic effects against death caused by S180 tumor cells were appreciably observed when mice were treated with ALP prior to tumor transplantation (conditions A and B). All mice died within 16 days after intraperitoneal inoculation of 10^5 S180 cells unless they were treated with ALP. However, significant prolongation of life spans and/or cure (60-day survival) were observed in tumor-bearing mice treated with ET-18-OMe analogs that carried a relatively longer alkyl chain at position 1 or dimethyl or monomethylaminoethylphosphate at position 3. No appreciable effect was observed with analogs having an alkyl chain shorter than C_{14} or an aminoethylphosphate at position 3. The antitumor activity against mouse syngeneic tumor MM46 was examined next (Table 1, condition D). In control mice (C3H/HeN) inoculated with 10^4 tumor cells, mortality was 100% within 20 days after transplantation. In contrast, intraperitoneal administration of these alkylphospholipids resulted in, at best, 60% cure of tumor-bearing mice (60-day survival). Derivatives having long alkyl chain (C_{22}) at position 1 or aminoethylphosphate at position 3 showed no appreciable activity.

An alkylphospholipid having an acetoacetyl moiety instead of O-methyl at position 2 showed appreciable antitumor activity against either S180 or MM46.

1,2-Di- and 1,3-di-*O*-alkyl-GPC carrying various chain length were also synthesized and tested. Among them, dioctyl-GPC (both α and β types) were found to have appreciable effects against S180 and MM46. Compounds having aliphatic chains either shorter or longer than dioctyl-GPC tended to lower the activity progressively. However, significant schedule dependency was observed; 1,2-di-*O*-nonyl- or decyl-GPC given intraperitoneally once on day 0 at a dose of 1 mg per mouse produced an appreciable increase in the life span of ICR mice bearing the S180 tumor (236% with dinonyl-GPC and 254% with didecyl-GPC), whereas 1,2-dioctyl-GPC showed weaker activity (195%).

Like ET-18-OMe, the acetoacetyl compound or 1,2-dioctyl-GPC could increase the life span of S180-bearing mice (Table 1, condition C) or inhibit the growth of tumor inoculated subcutaneously, even when ALP were injected only after tumor transplantation (Fig. 1). On the other hand, their direct cytotoxic effects on tumor cells were much weaker than that of ET-18-OMe; IC_{50} values against P388 leukemic cells were 34 μM with acetoacetyl compound, 145 μM with 1,2-dioctyl-GPC and 4 μM with ET-18-OMe.

Stereospecificity of antitumor action of ET-18-OMe and acetoacetyl compound. Intravenous injection of ET-18-OMe with *sn*-3 configuration retarded the subcutaneous growth of S180 cells effectively (Fig. 2A). Although the *sn*-1 enantiomer inhibited tumor growth, it was much less effective than the *sn*-3 compound. Racemic compound showed an intermediate activity. The treatment of mouse with racemic acetoacetyl compound was effective, while that with the *sn*-1 compound was not at all (Fig. 2B). No appreciable stereospecificity of the activity, however, was observed when the antitumor activity of ET-18-OMe was evaluated by prophylactic effect of intraperitoneal injection of ALP against death caused by S180 tumor cells (Table 2). Both the *sn*-1 and *sn*-3 compounds significantly increased the life span of the tumor-bearing mouse; no statistical difference was observed.

Even adverse stereospecificity was observed in a different set of experiments where antitumor effects of ALP against death of C3H/HeN mice caused by MM46 tumor cells propagated intraperitoneally were examined (Table 2). Three out of 5 mice survived 60 days after tumor inoculation when treated intraperitoneally with the *sn*-1 compound; the *sn*-3 compound did not increase the life span of MM46-bearing mice under the same conditions.

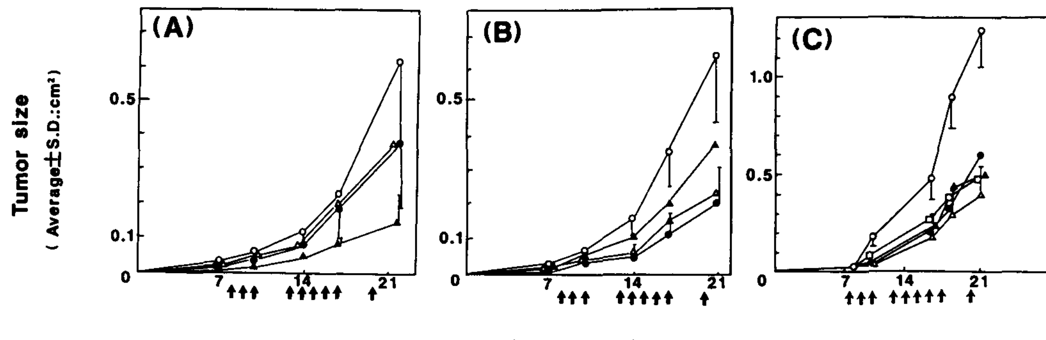


FIG. 1. Retardation of tumor growth by treatment of ALP. ICR mice (each group, 3 or 4 animals) subcutaneously inoculated with S180 (1×10^6 cells) were treated with ALP once a day on days 8, 9, 10, 13, 14, 15, 16, 17 and 20 as indicated by arrows. ALP of various doses were given per mouse per day. (A) ET-18-OMe, intravenous treatment: ○, control (nontreated); ▲, 30 μg (77%); △, 100 μg (39%); ●, 300 μg (33%). (B) Acetoacetyl compound, intravenous treatment: ○, control (nontreated); ▲, 30 μg (44%); △, 100 μg (53%); ●, 300 μg (55%). (C) Dioctyl-GPC, intraperitoneal treatment: ○, control (nontreated); ▲, 6 μg (55%); □, 20 μg (44%); △, 60 μg (60%); ●, 200 μg (50%). Numbers in parentheses represent percentage of retardation of average tumor weight on day 21 of treated mouse per control.

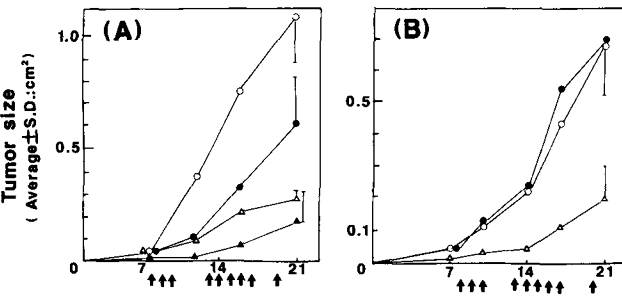


FIG. 2. Stereospecific retardation of the growth of S180 subcutaneously implanted by intravenous injection of ALP. (A) ET-18-OMe, 60 μg daily nine times as indicated by arrows. ○, Nontreated; ●, 3-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-1-phosphocholine (*sn*-1, 43%); ▲, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (*sn*-3, 71%); △, racemic 1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine (69%). (B) Acetoacetyl compound, 300 μg daily nine times as indicated by arrows. ○, Nontreated; ●, 3-*O*-octadecyl-2-*O*-acetoacetyl-*sn*-glycero-1-phosphocholine (*sn*-1, 101%); △, racemic 1-*O*-octadecyl-2-acetoacetyl-glycero-3-phosphocholine (55%). Numbers in parentheses represent percentage of retardation of average tumor weight on day 21 of treated mouse per control mouse.

TABLE 3

Induction of Cytostatic Peritoneal Exudate Cells In Vivo by ALPs

Agent tested	Dose (mg/mouse)	Radioactivity incorporated (dpm)[c] (Average ±SD)	
Experiment 1[a]			
None		74,571 ± 1,952	
Racemic ET-18-OMe	0.3	3,682 ± 1,403	(96)[d]
sn-3 ET-18-OME	0.3	46,667 ± 5,409	(37)[d]
sn-1 ET-18-OMe	0.3	4,942 ± 1,369	(93)[d]
Experiment 2[b]			
None		84,168 ± 1,186	
1,2-Dioctyl-GPC	1.0	15,729 ± 1,447	(81)[d]
1,2-Didecyl-GPC	1.0	73,525 ± 4,626	

[a]CDF1 mice (8 weeks) or [b]ICR mice (8 weeks) were injected intraperitoneally with ALP 4 days prior to sacrifice.

[c]Peritoneal exudate cells (4×10^5 cells/well) were incubated with target EL-4 cells (2×10^4 cells) for 24 hr at 37 C and the cytostasis was examined as described (16).

[d]Numbers in parentheses are the percent of cytostasis compared with peritoneal cells from untreated mice ($p < 0.001$).

TABLE 2

Antitumor Activity of ET-18-OMe Stereoisomers

Compound	In vivo: S180[a] T/C 0.5 mg	In vivo: S180[a] T/C 1 mg	In vivo: MM46[b] S/T	In vitro: HL-60, IC_{50}; μM[c]	In vitro: Hemolysis, HC_{50}; μM[d]
sn-3	175[e]		0/5	1.6	2.2
sn-1	146	176[f]	3/5	1.6	2.2

[a]Prophylactic effects against S180 were examined by a single intraperitoneal treatment of lipids (0.5 or 1 mg per mouse) as described in Table 1, condition A.

[b]Antitumor activity of lipids against MM46 was examined as described in Table 1, condition D.

[c]According to the method described (3), HL-60 cells (5×10^5 cells/ml) were incubated in D-MEM containing lipids for 24 hr in the absence of FCS. Viable cells were then determined by staining. IC_{50}: concentrations required for 50% growth inhibition.

[d]^{51}Cr-labeled human red blood cells (10^7 cells/ml) were treated with lipid for 30 min as described previously (20). HC_{50}: concentrations required for 50% hemolysis.

[e]$p < 0.01$.

[f]$p < 0.05$.

The tumor retardation, which was observed in vivo upon treatment with the *sn*-1 ET-18-OMe, might be partially due to modification of the host defense mechanism, since peritoneal exudate cells harvested from mice treated with the *sn*-1 compound showed appreciable cytostatic activity against EL-4 cells in vitro (Table 3). The peritoneal exudate cells, which were harvested from animals treated with the *sn*-3 compound, showed weaker inhibitory effect on growth of EL-4 cells. The ET-18-OMe showed direct cytotoxicity independent of its stereochemical configuration, since the *sn*-1 compound showed the same cytotoxicity as the *sn*-3 compound against HL-60 cells cultured in vitro and against erythrocytes.

Cytostasis and immunomodulaton by 1,2-dioctyl-GPC. Athough the antitumor activity of 1,2-dioctyl-GPC in vivo was stronger than that of dialkyl-GPC with longer aliphatic chains, such as 1,2-didecyl-GPC, the direct cytotoxic activity of the former against P388 cells in vitro was weaker than that of the latter (Table 1). On the other hand, the peritoneal exudate cells, which were harvested from mice treated with 1,2-dioctyl-GPC, showed appreciable cytostatic activity against EL-4 cells, whereas 1,2-didecyl-GPC was ineffective (Table 3).

Impairment of the antibody production against sheep red blood cells was observed in mouse-bearing, progressively growing S180 cells. Intraperitoneal injection of 1,2-dioctyl-GPC partially restored this immunodepression (Fig. 3), indicating that the dioctyl-GPC treatment modulates the immune response of the host. These results suggested that the antitumor activity of dioctyl-GPC seemed to be mainly through the enhancement of protective immunity of the host against tumor cells.

DISCUSSION

The acetoacetyl compound showed appreciable antitumor action against S180 and MM46 in vivo. The extent of its effectiveness was comparable to that of ET-18-OMe. In the acute toxicity study, the acetoacetyl compound was less toxic than ET-18-OMe; the LD_{50} values for the ICR mouse, when intraperitoneally administered once, were 104 mg/kg with acetoacetyl compound and 23 mg/kg with ET-18-OMe, respectively. An acetoacetyl compound caused much less necrosis in the tail vein, where the lipids were injected, than ET-18-OMe. The acetoacetyl compound is slightly less cytotoxic in vitro than ET-18-OMe; the IC_{50} value of the acetoacetyl compounds (racemic

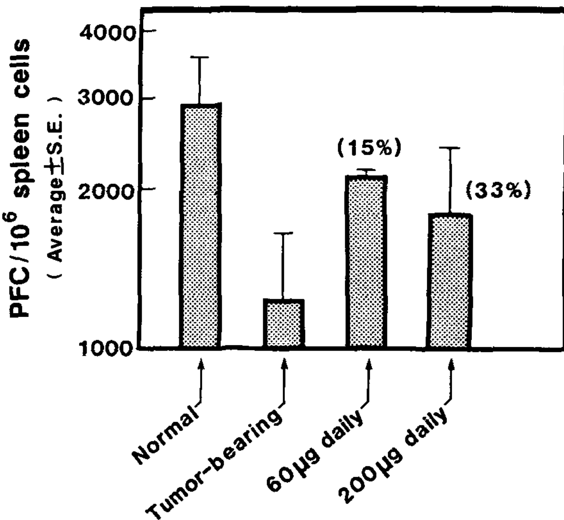


FIG. 3. Effect of 1,2-dioctyl-GPC on the antibody production in tumor-bearing mice. ICR mice bearing S180 tumor were treated daily with 1,2-dioctyl-GPC intraperitoneally six times at days 8, 9, 10, 11, 12 and 13. Antigen (sheep red blood cells) was intravenously injected at day 13, and the antibody production was assayed at day 17 as described previously (16). Each group contained six mice. Numbers in parentheses are the growth inhibition of S180 solid tumor due to lipid treatment.

compound) against HL-60 cells measured as described in Table 2 was 4.2 μM. This value was lower than the IC_{50} value against P388 (Table 1), which was examined in the presence of 10% FCS. The difference might be due to partial degradation of the acetoacetyl compound during incubation because, unlike ET-18-OMe, it was sensitive to phospholipase A_2 (data not shown). These results indicate that the acetoacetyl compound is one of the most attractive and promising antitumor alkyl phospholipids; further studies are desirable to include the elucidation of the molecular mechanism of the therapeutic activity of ALP.

ET-18-OMe as well as the acetoacetyl compound have some activity as platelet-activating factor (PAF) agonists, inducing platelet (18) or macrophage (10) activation, although their activity was considerably lower than that of PAF itself: the ED_{50} values to induce serotonin secretion from rabbit washed platelets were 2.1×10^{-5} (ET-18-OMe), 1.3×10^{-5} (acetoacetyl compound) and 8.1×10^{-10} M (PAF), respectively. The activation may be through specific receptors present on target cells, since the activation was blocked by CV-3988 (10), a well-known PAF antagonist (19). Like PAF, activation of either platelets or macrophages by ET-18-OMe was stereospecific; only the *sn*-3 compound was active (10). Similar stereospecificity was observed in their antitumor activity in vivo under certain experimental conditions, in which the tumor cells were propagated subcutaneously and ALP were injected intravenously through a remote route. The stereospecificity suggests that the observed antitumor effect might be mediated by activation of some host immune cells through their binding to PAF receptors.

The cells harvested from the peritoneal cavity could not be involved in these antitumor effects, since either cytotoxic or cytostatic activity could not be induced when PAF agonists, including ET-18-OMe, were incubated with mouse peritoneal exudate cells. Rat and mouse, unlike human, rabbit and guinea pig, do not have peripheral cells (platelets, polymorphonuclear cells, macrophages), which express specific binding sites for PAF on their surface (Table 4). In our preliminary experiments, however, specific binding of PAF could be detected on bone marrow cells, which include immature macrophages (Hayashi et al., data in preparation). We also detected specific binding sites for PAF on bone marrow cells derived from species other than mouse (guinea pig, rat and probably human [HL-60]). As illustrated in Figure 4, some PAF agonists may interact with bone marrow cells, most probably immature macrophages, inducing their differentiation or maturation. Macrophages matured by PAF agonists may express toxicity against tumor cells. This idea is consistent with previous findings by Munder et al. (19) that bone marrow-derived macrophages treated with racemic ET-18-OMe showed appreciable cytotoxicity against tumor cells. We suppose that the synthesis of pure *sn*-3 isomers of ALP might be important to obtain more effective antineoplastic compounds.

PAF did not show antitumor activity as observed with ET-18-OMe or acetoacetyl compound (data not shown), since it was toxic and induced shock, probably through a cardiovascular effect when injected systemically. The

TABLE 4

Specific Binding Sites for PAF

Species	Platelet	Polymorphonuclear leukocytes	Macrophage	Bone marrow cells
Mouse	No[a]	No[e]	No[e]	Yes[e]
Rat	No[a,b]	?	No[c,e]	Yes[e]
Guinea pig	Yes[a]	Yes[c]	Yes[e]	Yes[e]
Rabbit	Yes[a,b,c]	?	Yes[e]	Yes[e]
Human	Yes[a,b,c]	Yes[d]	Yes[e]	Yes[e,f]

[a]Ref. 24.
[b]Ref. 21.
[c]Ref. 22.
[d]Ref. 23.
[e]Hayashi et al., manuscript in preparation.
[f]We could detect specific PAF binding sites on human promyelocytic, leukemia HL-60 cells.

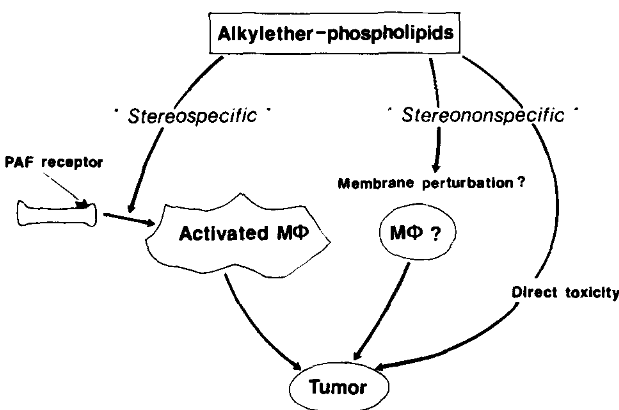


FIG. 4. Proposed mode of antitumor action of synthetic ALP.

antitumor effect might be observed only with PAF agonists that have rather weak cardiovascular effects. There is a possibility that the growth retardation of tumors subcutaneously implanted by PAF agonists may result from their effect on the vascular system developed inside or near the tumor. This possibility is, however, rather small, since a relatively small antitumor effect could be observed when the racemic ET-18-OMe was injected directly into the tumor (data not shown).

Direct cytotoxicity of ET-18-OMe against HL-60 cells and erythrocytes was not stereospecific. The antitumor activity of ET-18-OMe was independent of its stereochemical configuration when intraperitoneally administered to the ICR mouse prior to intraperitoneal inoculation of S180 cells. Adverse stereospecificity was even observed under certain experimental conditions. These findings suggest that the antitumor effect of ET-18-OMe might be primarily through an independent mechanism of a PAF agonist under some conditions. The effect might be due either to direct toxicity or to modification of some host cells. The latter possibility was suggested by findings that the antitumor effect was observable even when ET-18-OMe was injected four days prior to tumor inoculation and that cytostatic activity of peritoneal exudate cells was observed against EL-4 cells in vitro when harvested from mice treated with *sn*-1 ET-18-OMe. ALP analogs such as 1,2-dioctyl-GPC, which has relatively weak cytotoxic activity and is not a PAF agonist, also showed activity as biologic response modifiers since an intraperitoneal administration of 1,2-dioctyl-GPC induced cytostatic peritoneal cells, modulated antibody production in tumor-bearing mice and suppressed tumor growth of subcutaneously transplanted S180 cells. Thus we concluded that the stimulation of host system by ALP was not always through their binding to PAF receptors (Fig. 4).

We do not know whether the activity of dioctyl-GPC is stereospecific. The activity probably is not through binding to their specific receptors in cells, since even the β-type compound (1,3-dioctyl-GPC) showed the same activity as the α-type compound (1,2-dioctyl-GPC). The ET-18-OMe having *sn*-1 configuration as well as dioctyl-GPC may primarily cause nonspecific membrane perturbation, inducing activation of some host cells or suppressing tumor cell growth directly. We cannot explain why the *sn*-1 compound showed even higher antitumor activity in vivo under some conditions. Munder et al. previously reported that 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (*sn*-1) retarded the growth of MethA tumor cells much more effectively than a *sn*-3 enantiomer when given orally (1). One possibility is that the *sn*-1 compound is more resistant to metabolic degradation than the *sn*-3 enantiomer.

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Effect of Synthetic Phospholipids on Platelet Aggregation and Serotonin Release[1]

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1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor, PAF) is known to stimulate platelet aggregation and serotonin release in concentrations ranging from 10^{-10}–10^{-5} M. Since a variety of synthetic PAF analogues are potent antineoplastic agents in vitro and in vivo, it was the aim of this study to examine the PAF-like activity of 15 analogues, including 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) and a thioether analogue. In platelet-rich plasma from human blood, platelet aggregation and serotonin release were studied to compare the effects of PAF and the analogues. Platelet function was controlled by testing their response to adenosine diphosphate, arachidonic acid, collagen and epinephrine. Our results show that only PAF was able to induce platelet aggregation and serotonin release in concentrations from 10^{-9} to 10^{-5} M, whereas all the tested analogues up to a concentration of 10^{-3} M failed to induce these effects.
***Lipids 22*, 868–870 (1987).**

Platelet activating factor (PAF), a naturally occurring phospholipid, is known to be a very potent stimulator of rabbit and human platelet aggregation and secretion in vitro and in vivo (1–3). It first was discovered when IgE-sensitized rabbit basophils, after an immunological challenge, released PAF (4). Some years ago, the molecular structure of PAF was identified as 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (5). It has been shown that PAF is synthesized by a variety of cells, including polymorphnuclear neutrophils and mouse macrophages (6). There is evidence that PAF plays a major role in inflammatory reactions (7). PAF also is a very toxic compound. PAF-induced sudden death occurs at doses of 15 μg/kg when given to rabbits intravenously (8) resulting from the increased production of thromboxane A_2 by activated platelets (9), which leads to platelet aggregation, platelet degranulation and pulmonary thrombosis, among other pathophysiological reactions (8). PAF initiates irreversible platelet aggregation in a dose-dependent manner, reaching a maximum effect in a range of about 10^{-8} to 10^{-7} M (2,3). Platelet activation induced by PAF seems to take place independently of the cyclooxygenase pathway (10,11), is highly dependent on extracellular calcium and increases free intracellular calcium (12). In the last years, a variety of PAF analogues was synthesized and found to have antineoplastic properties. The close structural relationship between PAF and synthetic alkyl lysophospholipids made it necessary to examine whether these compounds might be able to cause PAF-like effects on human platelets.

MATERIALS AND METHODS

Materials. PAF (the hexadecyl derivative) and all of the synthetic PAF analogues were synthesized as described elsewhere (13,14). The compounds were dissolved in chloroform/methanol (1:9, v/v) and diluted in 0.9% NaCl containing 0.1% BSA [Sigma Chemical Co. (St. Louis, MO)]. Adenosine diphosphate, collagen, arachidonic acid and epinephrine were obtained from Bio-Data Corp. (Hatboro, PA). [^{14}C]Serotonin (5-hydroxy-[side chain-2-^{14}C]-tryptamine creatine sulfate, 57.4 mCi/mmol) was purchased from Amersham International (England).

Platelet aggregation and serotonin release. Venous blood was collected from young, healthy volunteers who had taken no drugs for at least two weeks; it was mixed with 3.8% (w/v) sodium citrate and blood (1:9, v/v). The anticoagulated blood was centrifuged at room temperature at 400 × g for 10 min, and the upper phase removed. The remaining blood was centrifuged at 1500 × g for 15 min to gain platelet-poor plasma (PPP). The platelet count for the platelet-rich plasma (PRP) was adjusted to 250,000 ± 50,000 per μl with PPP from the same sample. Testing was completed within two hr after blood collection. Platelet aggregation was studied at 37 C at a stirring speed of 1200 rpm using a Bio-Data four channel aggregometer (Model Pap-4, Colora). A volume of 270 μl PRP was incubated for two min at 37 C; then 30 μl of either adenosine diphosphate, arachidonic acid, collagen or epinephrine was added, and aggregation profiles were monitored. Provided the aggregation patterns of the samples were normal, the PAF sensitivity of the same platelets was tested in concentrations ranging from 10^{-9} to 10^{-6} M. Only when the response of each sample to the PAF stimulus was sufficient were aggregation profiles performed for 15 different synthetic PAF analogues up to a concentration of 10^{-3} M.

In a second set of experiments, platelets were labeled by incubating them with [^{14}C]serotonin (0.15 μCi/ml PRP) for 20 min at 37 C. Aggregation profiles were monitored as described above after adding either PAF or one of four selected PAF analogues. Aggregation and secretion were allowed to go on for five min. Subsequently, 70 μl of 6% (w/v) formaldehyde was added, the sample was put in an ice bath and centrifuged for one min at 12,000 × g. Serotonin release was calculated according the method of Holmsen and Dangermaier (15).

RESULTS AND DISCUSSION

As shown in Figure 1, PAF itself stimulates irreversible aggregation of human platelets in the range of 5 × 10^{-8} M

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Abbreviations: PAF, platelet activating factor; PPP, platelet-poor plasma; PRP, platelet-rich plasma; BSA, bovine serum albumin; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; BM 41.440, 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine.

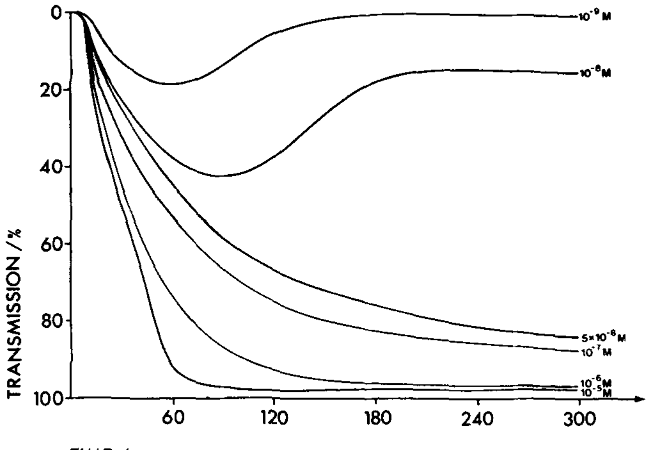

FIG. 1. PAF-induced platelet aggregation in human PRP.

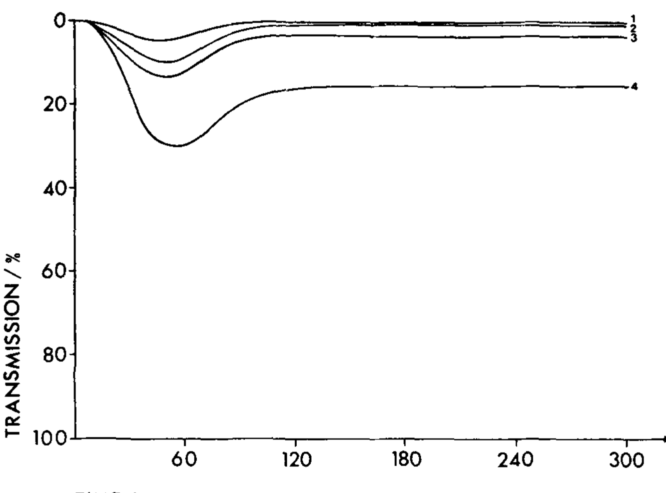

FIG. 2. Platelet aggregation of PAF analogues (10^{-3} M) in human PRP. 1: Hexadecylphosphocholine; 2: 1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; 3: 1-*O*-Hexadecyl-propandiol-2-phosphocholine; 4: 1-Hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine.

TABLE 1

Investigated PAF Analogues

1. 3-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-1-phosphocholine
2. 1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine
3. 1-*O*-Octadecyl-*sn*-glycero-3-phosphocholine
4. 3-*O*-Octadecyl-*sn*-glycero-1-phosphocholine
5. 1-*O*-Hexadecyl-*sn*-glycero-3-phosphocholine
6. 3-*O*-Hexadecyl-*sn*-glycero-1-phosphocholine
7. 1-*O*-Octadecyl-propandiol-3-phosphocholine
8. 1-Hexadecyl-propandiol-2-phosphocholine
9. Hexadecylphosphocholine
10. Octadecylphosphocholine
11. 1-*O*-Palmitoyl-*sn*-glycero-3-phosphocholine
12. 1-Octadecyl-propandiol-3-phospho-(*N,N,N*-trimethylamino)-hexanol
13. 1-*O*-Hexadecyl-*sn*-glycero-3-phospho-(*N,N,N*-trimethylamino)-hexanol
14. Oleyl-phospho-*N,N,N*-(trimethylamino)-hexanol
15. 1-Hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine

TABLE 2

Secretion of ^{14}C-Serotonin after Stimulation with PAF and Four Selected PAF Analogues

Compound	Concentration (mol/l)	Serotonin release (% accumulated ^{14}C-serotonin[a]
1-*O*-Hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (PAF)	10^{-5}	53.13 ± 10.59 (6)
Hexadecylphosphocholine	10^{-3}	2.39 ± 2.01 (5)
1-*O*-Hexadecyl-propandiol-2-phosphocholine	10^{-3}	3.83 ± 1.37 (5)
1-Hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine	10^{-3}	4.45 ± 1.70 (5)
1-*O*-Octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine	10^{-3}	5.19 ± 0.63 (5)

[a]Values are means ±SD. No. of experiments are shown in parentheses.

to 10^{-5} M, whereas reversible aggregation still could be detected at 10^{-9} M.

In contrast, all PAF analogues tested were without effect on platelet aggregation up to concentrations of 10^{-4} M. At 10^{-3} M, a minor reversible aggregation occurred with some analogues (Fig. 2), which most likely results from an unspecific effect on platelet membranes. This result also is obtained for the compounds shown in Figure 2 and for those summarized in Table 1. The same structures also were ineffective with respect to serotonin release when tested up to 10^{-3} M (Table 2) under conditions where PAF initiated release of more than 50% of platelet [^{14}C]serotonin at 10^{-5} M.

The observation of Wykle et al. (16) that 1-*O*-hexadecyl-2-*O*-methyl-GPC is a weak agonist for rabbit platelets does not apply to human platelets. This apparent discrepancy most likely reflects the difference in the species used as a platelet source.

It appears that the existence of a short acyl ester in the 2-position of the glycerol moiety represents a minimum requirement for the 1-*O*-alkyl-glycero-phospholipids in order to exert a physiological response in human platelets. This is in accordance with similar conclusions made by others (17). Because phospholipid analogues of platelet activating factor (PAF) represent a new approach to cancer chemotherapy, it seems important that no interference with the physiological action of PAF is to be expected as long as this minimal structural requirement is considered.

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Immunomodulatory and Therapeutic Properties of Alkyl Lysophospholipids in Mice[1]

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This paper describes the immunomodulatory and therapeutic properties of the alkyl lysophospholipids [ALP; 1-*O*-octadecyl-2-*O*-*rac*-glycero-3-phosphocholine (ET-18-OCH_3)]. ALP was able to activate macrophages both in vitro and in vivo as well as to act as an immunoadjuvant for syngeneic tumor vaccines. However, ALP appeared to be transferred, at least in part, to the macrophage membrane, and some of the tumoricidal macrophage-activating properties seem to be associated with the direct cytotoxic effect of membrane-released ALP. ALP also had some therapeutic activity for experimental and spontaneous metastases, requiring administration three but not two times weekly at near-toxic doses; this suggests that at least some of its therapeutic activity is due to direct cytotoxicity.
Lipids 22, 871–877 (1987).

It has been reported that some immunopotentiators that stimulate host defense mechanisms against infections and tumors have a common biochemical effect. Following endocytosis by macrophages, these immunomodulatory compounds may activate a phospholipase that degrades phosphatidylcholine and phosphatidylethanolamine to the lyso derivatives and free fatty acids (1–5). Lysophosphatidylcholine, for example, was found to be active as an immunopotentiator. Naturally occurring lysophospholipids have been found to be humoral and cellular immunomodulatory agents (5–7). Since these compounds are rapidly metabolized, synthetic alkyl derivatives were developed that have extended half-lives and do not serve as substrates for cellular enzymes (8). The alkyl lysophospholipids (ALP) have been shown to have several effects on the immune system. These compounds have been described as a new class of biological response modifiers (BRM) that inhibit the growth (5,9) and metastasis (10) of syngeneic experimental mouse tumors and experimental rat tumors (11,12). Studies have indicated that the therapeutic activity of ALP may be mediated by augmenting the cytotoxic properties of macrophages (5,9,10). In addition, some ALP can destroy leukemic (13–15) and tumor cells (16,17), indicating a direct cytotoxic effect. Other ALP have been shown to induce differentiation of leukemic blast cells (18) or to counteract tumor cell invasion (19).

We have undertaken an evaluation of several BRM (20,21), including ALP, prior to considering them for clinical trials. Using a systematic approach, we initially examined the effects of BRM on the effector cell populations of normal animals in vitro. Subsequent studies of in vivo immunomodulation provided data on BRM-host interactions, including degradation, inhibition and the requirement for BRM metabolism or cellular cooperation resulting in BRM-induced immunomodulation, as well as information on dosage and toxicity. On the basis of these preliminary investigations, a BRM's therapeutic properties are studied in a quantitative and qualitative manner. We report here our studies of the immunomodulatory properties of ALP and provide information on its therapeutic efficacy.

MATERIALS AND METHODS

Animals. Specific-pathogen-free male C57BL/6 mice (H-2^b) and C3H/HeN (MTV-) mice (H-2^k), 3 or 4 wk of age, were obtained from the Animal Production Area of the National Cancer Institute–Frederick Cancer Research Facility (Frederick, MD).

Tumors. These studies used the radiation-induced fibrosarcoma UV-2237 (22), syngeneic to the H-2^k mouse, and UV-2237 Cl 46, a regressor clone (23) obtained from UV-2237. Lymphomas used included the methylcholanthrene-induced mastocytoma P815 (24), syngeneic to the DBA/2 mouse (H-2^d), and the Moloney virus-induced lymphoma YAC-1 (25) of A/SN (H-2^a) origin. Therapy studies used the metastatic melanoma variant B16-BL6 (26), which was selected in vitro from the B16 melanoma, a spontaneous tumor from a C57BL/6N mouse. The fibrosarcoma and melanoma were maintained as monolayers in minimum essential medium supplemented with 5% fetal bovine serum, glutamine, sodium pyruvate, twofold vitamins and nonessential amino acids (complete minimum essential medium). The P815 and YAC-1 tumor cell lines were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and the same medium supplements added to Eagle's minimum essential medium. All cell lines were free of *Mycoplasma* and pathogenic murine viruses. The media, supplements and serum contained less than 0.1 ng/ml of endotoxin, as assessed by the *Limulus* lysate assay (21).

Agents. ALP were provided by Dr. W. E. Berdel of the Technical University, Munich, FRG. Polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose [poly (I,C)-LC] was donated by Dr. Hilton Levy (National Institute of Allergy and Infectious Diseases, Frederick, MD) and the thymosin fraction 5 was obtained from Hoffmann-La Roche, Inc. (Nutley, NJ).

Technical approach. The experiments were repeated at least three times, and any study in which either the negative or positive control did not function was performed again. In vitro assays were analyzed with the

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Abbreviations: ALP, alkyl lysophospholipids; BRM, biological response modifiers; poly (I,C)-LC, polyinosinic-polycytidylic acid complexed with poly-L-lysine and carboxymethylcellulose; MLR, mixed lymphocyte reaction; MLTR-CMC, mixed lymphocyte tumor response–cell-mediated cytotoxicity; HBSS, Hanks' balanced salts solution; NK, natural killer; MTD, maximum tolerated dose.

paired Student's t-test, and the Mann-Whitney U-test was used to compare the median number of metastases.

Mixed lymphocyte reaction (MLR). Responder spleen cells were admixed with irradiated stimulator spleen cells in flat-bottom, 96-well plates in culture medium containing various doses of ALP or thymosin fraction 5, which served as a positive control. The culture medium described by Click et al. (27) was supplemented with 0.5% murine serum. The cultures were pulse-labeled with [^{3}H]thymidine (1 μCi/well) for 24 hr before harvest, which was performed with an automatic cell harvester 4 or 5 days after culture initiation (21).

Effector cells stimulated in an allogenic mixed lymphocyte tumor response-cell-mediated cytotoxicity (MLTR-CMC) assay. In the MLTR-CMC assay, effector cells were stimulated by culturing C3H/HeN (H-2^k) splenic lymphocytes with irradiated P815 (H-2^d) tumor cells. Experimental cultures of responder and stimulator cells contained various concentrations of ALP, whereas control cultures included spleen cells cultured in normal medium or in medium supplemented with ALP. After a 5-day incubation, the effector lymphocytes were washed several times; lymphocyte viability was assessed and the cytotoxic properties were determined in a 4-hr ^{51}Cr-release assay with P815 targets. The effector cell induced was specific for P815 and sensitive to anti-Thy antiserum but not to anti-asialo GM-1 antiserum. Cytotoxicity was calculated with the following formula:

$$\% \text{ of cytotoxicity} = \frac{\text{cpm released by lymphocytes} - \text{cpm released spontaneously}}{\text{Total Triton-released cpm}}$$

Induction of tumor-specific cytotoxic T lymphocytes. Mice were immunized intradermally (i.d.) with 1 $\times$ 10^6 irradiated, collagenase-dissociated UV-2237 tumor cells, with or without an adjuvant, by injecting 0.05 ml of Hanks' balanced salts solution (HBSS) containing 2 $\times$ 10^5 cells into five discrete sites. Experimental vaccines consisted of tumor cells admixed with HBSS or one of several doses of ALP. Control vaccines consisted of HBSS or ALP without tumor cells. Target cells for the cytotoxic T lymphocyte assays were added to a flat-bottom, 96-well plate in 0.1 ml of complete minimum essential medium containing 5% fetal bovine serum and 5000 viable tumor cells radiolabeled with [^{75}Se]methionine. Ten days after immunization, specific effector cells were added in triplicate at various effector-to-target cell ratios. The cocultures were incubated at 37 C for 18 hr and centrifuged for 3 min. Aliquots (0.1 ml) were removed, and the amount of radioactivity was determined. The percentage of cytotoxicity was calculated according to the following formula:

$$\% \text{ of cytotoxicity} = \frac{\text{cpm released in cultures with effector cells} - \text{cpm released spontaneously}}{\text{Total Triton-released cpm} \times 100}$$

Assay of macrophage-mediated cytotoxicity. Thioglycollate-elicited peritoneal exudate macrophages were collected, and the macrophage cytotoxicity assay was performed as previously described (20). In brief, macrophage suspensions were plated into a 96-well plate and incubated for 2 hr, and nonadherent cells were removed. The monolayers were incubated with control medium or an activating agent for 24 hr, after which the medium was removed and replaced with medium containing 1 $\times$ 10^4 IUdR-radiolabeled B16-BL6 target cells. In experiments using in vivo-activated macrophages, mice were given intraperitoneal (i.p.) injections of HBSS, poly (I,C)-LC (which served as a positive control), or ALP. Twenty-four hr later, peritoneal cells were harvested, adherence-purified and cocultured with radiolabeled target cells. The cytotoxicity assays were terminated 72 hr after the addition of target cells; the monolayers were washed, viable adherent cells were lysed and radioactivity was monitored. Under the conditions of our assay, normal (untreated) macrophages were not cytotoxic to neoplastic cells. The cytotoxic activity of the macrophages was calculated with this formula:

$$\% \text{ of cytotoxicity} = \frac{100 \times \text{cpm in target cells cultured with normal macrophages} - \text{cpm in target cells cultured with test macrophages}}{\text{cpm in target cells cultured with normal macrophages}}$$

Augmentation of natural killer (NK) cell activity. The ability of ALP to augment NK activity in vitro was determined by incubating spleen cells for 24 hr with control medium or medium containing various doses of ALP. The in vivo augmentation of NK activity was assessed after intravenous (i.v.) injection of various doses of ALP or HBSS, which served as the negative control. Three-wk-old C3H/HeN mice were routinely used for NK augmentation studies since they have a low initial level of NK activity. NK cell activity was assessed in a 4-hr ^{51}Cr-release assay using YAC target cells, as previously described (20).

Therapy of established metastases. Eight-wk-old syngeneic C57BL/6N mice were given i.v. injections of a single cell suspension of 4 $\times$ 10^4 in vitro-propagated B16-BL6 melanoma cells in calcium/magnesium-free HBSS. The schedule of therapeutic injections varied for each experiment. Therapy was continued for 4 wk; experimental mice that were alive 1 wk after the last control animal had died were killed and necropsied. Determinations of therapeutic efficacy were based on survival of the mice and the extent of pulmonary metastasis as analyzed by the Mann-Whitney U-test.

The therapeutic efficacy of ALP was also evaluated against spontaneous metastases from B16-BL6 melanoma cells. Eight-wk-old syngeneic mice were inoculated in a posterior footpad with 5 $\times$ 10^4 B16-BL6 melanoma cells in 0.05 ml of calcium/magnesium-free HBSS. When the primary tumor achieved a diameter of 0.8 to 1 cm, the tumor-bearing leg was resected at midfemur to include the popliteal lymph node. Twenty-four hr later, we initiated therapy using a protocol of injections three times per week for 4 wk. Necropsies were performed 1 wk after the last injection. The lungs were fixed, and the extent of spontaneous pulmonary metastasis was determined by counting the metastases using a dissecting microscope.

RESULTS

Augmentation of NK cell activity. Incubation of spleen cells with ALP at doses of 1.0 to 0.00001 μg/ml did not significantly augment NK cell activity in vitro (Table 1). However, the positive control, poly (I,C)-LC, did activate NK cells in vitro at 5 μg/ml. In addition, ALP is inactive in promoting NK cell augmentation in vivo at doses between 10 and 0.001 μg/animal (results not shown) when measured 1 or 3 days after injection.

TABLE 1

Effect of ALP on NK Cell Activation In Vitro[a]

Agent	Concentration (μg/ml)	Percent cytotoxicity[b] 50:1	25:1	12:1	6:1
Medium	—	17	9	2	1
Poly (I,C)-LC	5.0	66[c]	49[c]	32[c]	14[c]
ALP	0.00001	19	11	3	0
ALP	0.0001	31[c]	14	5	3
ALP	0.001	23	12	6	1
ALP	0.01	23	10	7	4
ALP	0.1	16	6	3	1
ALP	1.0	24	10	5	2

[a]Spleen cells from C3H mice were incubated in vitro for 24 hr with complete medium (negative control), poly (I,C)-LC (positive control) or various doses of ALP. The effector cells were washed and their cytotoxic potential was examined in a 4-hr ^{51}Cr-release assay with YAC tumor cell targets.

[b]Percent cytotoxicity was determined at effector-to-target cell ratios of 50:1, 25:1, 12:1 and 6:1.

[c]Significant increase in cytotoxicity as determined by the paired Student's t-test ($p \leq 0.05$).

TABLE 2

Effect of ALP on Mixed Lymphocyte Reaction (MLR)[a]

Agent	Dose (μg/ml)	Responder cells	Responder cells + syngeneic stimulation	Responder cells + allogenic stimulation
Medium	—	1079	1153	2043
Thymosin fraction 5	100.0	4319[b]	4689[b]	7713[b]
ALP	0.0001	1685[b]	1801[b]	3323[b]
ALP	0.001	1727[b]	1700[b]	3541[b]
ALP	0.01	1846[b]	2030[b]	3693[b]
ALP	0.1	2183[b]	2347[b]	4123[b]
ALP	1.0	135[b]	158[b]	309[b]

[a]Irradiated C57BL/6 mice stimulator spleen cells were admixed with C3H mouse responder spleen cells at a stimulator-to-responder cell ratio of 1:10. The cultures were incubated with medium alone (negative control), thymosin fraction 5 (positive control) or various concentrations of ALP for 4 days before harvest and were pulsed with 1 μCi of [^{3}H]thymidine for 24 hr. Quadruplicate cultures were performed.

[b]Significant difference in cpm compared to medium control, as determined with Student's t-test ($p \leq 0.01$).

MLR. ALP was added to allogenic MLR cultures, which were initiated at a suboptimal stimulator-to-responder ratio (1:8) to demonstrate maximum immunomodulation (29). As a positive control, spleen cells were cocultured with thymosin fraction 5 (Table 2), which induced a significant increase in the cpm incorporated into responder lymphocytes following 4 or 5 days of coculture. Coculturing spleen cells in the presence of ALP (Table 2) significantly increased the incorporation of [^{3}H]thymidine into responder cells in the presence or absence of stimulator cells. At 4 days following coculture, ALP produced a stimulator index of 3.1 to 3.8 at concentrations ranging from 0.1 to 0.0001 μg/ml. However, at a concentration of 1 μg/ml, ALP was significantly toxic to the spleen cells, such that the coculture resulted in background levels of [^{3}H]thymidine into responder cells in the presence or absence of allogenic stimulator cells, i.e., a slight "blastogenic" activity was observed.

MLTR-CMC. ALP was also tested in an allogenic MLTR-CMC assay. These assays were performed at a suboptimal stimulator-to-responder ratio (1:300) to permit the demonstration of immunostimulation (28). At ALP concentrations ranging from 1 to 0.0001 μg/ml, cytotoxicity was not significantly increased compared to levels of activity observed with cultures in normal medium (Table 3). The positive control in this experiment, thymosin fraction 5, did significantly stimulate cytotoxic effector cell activity. The percent viability of the cells following 5 days of coculture ranged from approximately 60% to 87% in either the presence or absence of stimulator cells. However, at 1 μg/ml of ALP, the percent viability was reduced to 33% (with stimulator cells) or 36% (without stimulator cells). In addition, the number of cells per culture ranged from 6.4 to 12 $\times 10^6$ cells per culture following 5 days of coculture, whereas this number was reduced to 1.2 to 1.6 $\times 10^6$ cells per culture at 1 μg/ml of ALP. Thus, as in the MLR, ALP at 1 μg/ml had significant toxicity against spleen cells following 4 or 5 days of coculture. This was also seen in the effector cell function of spleen cells cultured with stimulator cells in the presence of ALP at 1 μg/ml. Control cultures with stimulator cells had 40% cytotoxicity at an effector-to-target cell ratio of 50:1, whereas the level of cytotoxicity was 0% following coculture with 1 μg/ml of ALP. Thus, on the basis of a number of parameters, including [^{3}H]thymidine incorporation, effector cell activity, cell numbers and percent viability, ALP at doses equal to 1 μg/ml were judged to be toxic to lymphocytes.

Specific adjuvant activity of ALP. The immunoadjuvant activity of ALP was assessed by examining their effect on the in vivo generation of splenic cytotoxic T lymphocyte activity. Normal syngeneic mice were immunized with an i.d. injection of lethally irradiated tumor cells obtained by the collagenase-DNase dissociation of subcutaneous tumors. Spleen cells from mice immunized with tumor cells alone had significantly increased splenic cytotoxic activity compared to control spleen cells (Table 4). Adding 0.01 and 0.001 μg of ALP per site to the tumor cell baccine significantly increased the level of cytotoxic effector cell activity compared to the vaccine alone. It was observed that ALP did not stimulate the cytotoxic T lymphocyte effector cell activity in the absence of syngeneic tumor vaccine.

Macrophage activation. The ability of ALP to activate

TABLE 3

Effect of ALP on In Vitro Generation of Alloreactive Cytotoxic T Lymphocytes (MLTR-CMC)[a]

Agent	Dose (μg/ml)	Stimulator cells	No. cells (× 10⁶)	Percent viability (day 5)	Percent cytotoxicity[b] 50:1	25:1	12:1	6:1
Medium	—	—	9.8	68	0	0	0	0
Thymosin fraction 5	100	—	11.0	78	3	1	0	0
ALP	0.0001	—	9.0	78	0	0	0	0
ALP	0.001	—	9.7	65	0	0	0	0
ALP	0.01	—	6.3	60	0	0	0	0
ALP	0.1	—	6.4	76	0	0	0	0
ALP	1.0	—	1.6	36	0	0	0	0
Medium	—	+	9.4	87	40	25	14	9
Thymosin fraction 5	100	+	12.0	80	67[c]	59[c]	46[c]	28[c]
ALP	0.0001	+	9.8	82	32	21	11	6
ALP	0.001	+	9.3	86	33	20	13	7
ALP	0.01	+	9.4	76	30	14	6	4
ALP	0.1	+	9.3	76	42	25	15	8
ALP	1.0	+	1.2	33	0[c]	2[c]	0[c]	0[c]

[a]Allogenic C3H mouse spleen cells were cocultured with or without irradiated P815 tumor cells for 5 days in medium containing 0.5% normal murine serum (negative control). Experimental cultures were incubated with thymosin fraction 5 (positive control) or ALP. After culture, the cytotoxic activity of the spleen cells was tested in a 4-hr ^{51}Cr-release assay using P815 target cells.

[b]Cytotoxicity was determined on day 5 at effector-to-target cell ratios of 50:1, 25:1, 12:1 and 6:1.

[c]Significant difference in cytotoxicity compared to medium control as determined with Student's t-test ($p \leqslant 0.01$).

TABLE 4

Adjuvant Activity of ALP and Development of Syngeneic Cytotoxic T Lymphocytes[a]

Agent	Dose (per site)	UV-2237 vaccine	Percent cytotoxicity[b] 200:1	100:1	50:1	25:1
HBSS	0.05ml	—	14	7	3	5
HBSS	0.05 ml	+	58[c]	32[c]	16[c]	8
Poly (I,C)-LC	1.0 μg	+	49	39	35	16
Poly (I,C)-LC	2.0 μg	+	52	35	18	11
Poly (I,C)-LC	5.0 μg	+	24	18	7	5
ALP	0.1 μg	+	40[c]	28[c]	17[c]	10
ALP	0.01 μg	+	73[c,d]	45[c,d]	20[c,d]	12[c,d]
ALP	0.001 μg	+	72[c,d]	30[c]	17[c]	11
ALP	0.1 μg	—	10	2	6	4

[a]C3H mice were immunized with collagenase-DNase-dissociated syngeneic UV-2237 tumor cells (5 × 10⁶ cells total in 5 i.d. injections) in HBSS or ALP. The adjuvant was administered at time 0 as an admixture. Eight days after immunization, effector cell activity was examined in a 24-hr radiorelease assay with [^{75}Se]methionine-labeled Cl-46 cells; these were obtained from clonal subpopulations of UV-2237 and more sensitive to cytotoxic T cell activity than the parent tumor (spontaneous release, 26%). Each group contained 3 mice.

[b]Cytotoxicity was determined at effector-to-target cell ratios of 200:1, 100:1, 50:1 and 25:1.

[c]Significant increases compared to sham-treated animals, as determined with the paired Student's t-test ($p \leqslant 0.01$).

[d]Significant change in cytotoxicity compared to tumor vaccine without adjuvant, as determined with the paired Student's t-test ($p \leqslant 0.01$).

macrophages in vitro was studied by incubating C57BL/6N thioglycollate-elicited macrophages with ALP for 24 hr in vitro. Cytotoxicity was assessed using syngeneic [^{125}I]IUdR-labeled B16-BL6 target cells in a 72-hr assay. ALP appeared to activate macrophages to become cytotoxic over a range from 500 to 0.0001 μg/ml (Table 5). However, ALP at concentrations greater than 10 μg/ml produced cytotoxic effects independent of macrophage activation. This high-dose toxicity is evident in Table 6, which presents the results of an assay from which macrophages were omitted. Doses of ALP from 500 to 0.01 μg/ml were cytotoxic for target cells in the absence of macrophages when M109 cells were used as a substitute monolayer for the macrophages (Table 6). These results strongly suggest a direct cytotoxic effect, which is increased in the presence of a monolayer of cells. Thus, much of the macrophage activation that has been reported in vitro, as assayed by tumoricidal activity, may be associated with nonspecific adsorption of ALP to either the tissue culture plastic or the cellular monolayer. ALP administered in vivo did, however, induce macrophage-mediated cytotoxicity, as shown in Table 7. In this study, poly (I,C)-LC served as the positive control. ALP induced macrophage tumoricidal activity at doses from 10 to 0.1 mg/kg when administered i.p., and peak activation was observed at 1 mg/kg. Thus, unlike the in vitro incubation of macrophages, the ability of ALP to activate macrophages in vivo was clearly demonstrated.

Therapy of experimental and spontaneous metastases. Experimental and spontaneous metastasis treatment models were used to investigate the therapeutic potential of ALP. In the experimental metastasis model, therapy was initiated 3 days after the i.v. injection of B16-BL6 tumor cells and was continued for 4 wk (Table 8). At the beginning of therapy, numerous microscopic pulmonary tumor nodules were histologically evident. Injecting ALP i.v. twice a week did not significantly reduce the number of experimental metastases at any dose study. By contrast, the positive control, poly (I,C)-LC, did produce a significant therapeutic effect. It

TABLE 5

In Vitro Activation of Thioglycollate-Elicited Peritoneal Macrophages by ALP[a]

Agent	Dose (μg/ml)	cpm (Day 3)	Percent cytotoxicity
Medium	—	1311	
ALP	500.0	71	94.5[b]
ALP	100.0	918	29.9[b]
ALP	10.0	918	29.9[b]
ALP	1.0	1061	19.0[b]
ALP	0.1	602	54.0[b]
ALP	0.01	679	48.2[b]
ALP	0.001	734	44.0[b]
ALP	0.0001	719	45.1[b]
ALP[c]	500.0	245	81.3[b]
ALP	100.0	990	24.4[b]
ALP	10.0	1360	0
ALP	1.0	1360	0
ALP	0.1	1378	0
ALP	0.01	1404	0
ALP	0.001	1300	0.8
ALP	0.0001	1240	5.4

[a]Thioglycollate-elicited peritoneal macrophages were incubated for 24 hr with medium or ALP at various doses. [^{125}I]IUdR-labeled B16-BL6 tumor cells were plated onto the peritoneal macrophage monolayers in triplicate, incubated for 72 hr and assayed for cytotoxicity.

[b]Significantly increased cytotoxicity compared to medium control, as determined with paired Student's t-test ($p \leqslant 0.05$).

[c]To determine nonspecific adsorption of ALP to the plastic tissue culture plates and resultant toxicity to tumor cells, wells were incubated with the same doses of ALP for a 24-hr period and then washed in the same manner as wells containing macrophages and ALP.

TABLE 6

In Vitro Activation of Thioglycollate-Elicited Peritoneal Macrophages by ALP[a]

Agent	Dose (μg/ml)	cpm (Day 3)	Percent cytotoxicity
Spontaneous release	—	1614	—
HBSS	—	1654	0
ALP	500.0	94	94.3[b]
ALP	100.0	406	75.7[b]
ALP	10.0	1400	16.5[b]
ALP	1.0	1504	10.3[b]
ALP	0.1	1336	20.3[b]
ALP	0.01	1512	9.8[b]
M109 alone	—	1701	−2.8
ALP[c]	500.0	213	87.2[b]
ALP	100.0	209	87.5[b]
ALP	10.0	942	43.8[b]
ALP	1.0	342	79.,6[b]
ALP	0.1	362	78.4[b]
ALP	0.01	277	83.4[b]

[a]Thioglycollate-elicited peritoneal macrophages were incubated for 24 hr with medium, poly (I,C)-LC (positive control) or ALP at various doses. [^{125}I]IUdR-labeled B16-BL6 tumor cells were plated onto the peritoneal macrophage monolayers in triplicate, incubated for 72 hr and assayed for cytotoxicity.

[b]Significantly increased cytotoxicity compared to medium control, as determined with the paired Student's t-test ($p \leqslant 0.05$).

[c]M109 tumor cells (an adherent cell line) were attached to the wells of the 96-well plate and coincubated with ALP for 24 hr. This allowed us to determine a control value of ALP adherence to the cell monolayer.

TABLE 7

In Vivo Activation of Peritoneal Macrophage-Mediated Cytotoxicity by ALP[a]

Agent	Dose (mg/kg)	Macrophages per animal ($\times 10^5$)	cpm (Day 3)	Percent cytotoxicity
Spontaneous release		—	1059	—
HBSS		1.9	1030	2.7
Poly (I,C)-LC	2.5	2.5	678	35.9[b]
ALP	10.0	1.2	895	15.4[b]
ALP	1.0	2.2	541	48.9[b]
ALP	0.1	2.1	909	14.1[b]

[a]C57BL/6 mice (n = 5/group) received i.p. injections of poly (I,C)-LC or ALP. Twenty-four hours later, peritoneal exudate cells were harvested. The macrophages were purified by adherence and used as effector cells with [^{125}I]IUdR-labeled B16-BL6 tumor cells in a 72-hr assay.

[b]Significant decrease in cpm compared to HBSS control Student's t-test ($p \leqslant 0.05$).

TABLE 8

Effect of ALP on Experimental Metastases[a]

Agent	Dose (mg/kg)	Median survival (days)	Median no. of metastasis (range)
HBSS	—	32	>300 (180->300)
Poly (I,C)-LC	1.5	34[b]	32[c] (24-292)
ALP	100.0	21[d]	240 (28->300)
ALP	10.0	28	>300 (208->300)
ALP	1.0	32	>300 (72->300)

[a]Syngeneic C57BL/6 mice (n = 10/group) received i.v. injections of B16-BL6 tumor cells (2.5×10^4). Three days later, therapy was initiated with twice weekly i.v. injections for 4 weeks. The study was terminated on day 70.

[b]Significant prolongation in survival according to the Kruskal-Wallis analysis ($p < 0.05$).

[c]Significant reduction in the median number of pulmonary colonies according to the Mann-Whitney U-test ($p < 0.01$).

[d]Fifty percent of the animals died of ALP toxicity, with a resultant median survival time of 21 days.

should be noted that the injection of ALP at 100 mg/kg significantly reduced the survival of these mice. Thus, even doses of ALP greater than the maximum tolerated dose (MTD) had no significant therapeutic activity when administered twice a week.

In a second study in which ALP was injected i.v. three times a week for 4 wk, we found a significant therapeutic response in animals with spontaneous metastases (Table 9). Mice began the therapeutic protocol 3 days after the resection of the primary B16-BL6 footpad tumor. Those that received excipient (HBSS) injections developed a median of 90 spontaneous metastases, and a significant reduction in the median number of lung nodules was observed in mice treated with ALP at 10 and 25 mg/kg. At 0.5 mg/kg, the positive control, poly (I,C)-LC, also produced a significant level of therapeutic

TABLE 9

Effect of ALP on Spontaneous Metastases[a]

Agent	Dose (mg/kg)	Median no. of metastases (range)	Median survival (days)	Percent cure[b]
HBSS	–	90 (0->300)	31 (28-521)	10
Poly (I,C)-LC	0.5	27[c] (0->300)	37[d] (31-521)	20
ALP	50.0	32 (0->300)	35[d] (25-521)	10
ALP	25.0	13[c] (0-205)	43[d] (22-521)	40
ALP	10.0	11[c] (0-65)	31 (25-521)	28

[a]Mice (n = 10/group) received injections of 5 × 10^4 B16-BL6 tumor cells into a hind footpad. When the tumors reached a diameter of 0.8-1.0 cm, the tumor-bearing limb was resected at midfemur to include the popliteal lymph node. Therapy was initiated 3 days later and consisted of i.v. injections twice weekly for 4 weeks. Necropsies were performed 22 days after the last injection.

[b]Animals that were grossly tumor-free 52 days (censored) after tumor resection.

[c]Probability of no differences as determined using the Mann-Whitney U-test.

[d]Significant prolongation in survival according to the Kruskal-Wallis analysis ($p < 0.05$).

activity. In contrast to the results, when ALP was administered twice a week, if it has been administered three times a week, a significant therapeutic response was observed. Interestingly, a significant prolongation of survival was observed in mice receiving 25 or 50 mg/kg of ALP with significantly greater therapeutic activity observed at 25 mg/kg ($p = 0.041$ by the Kurskal Wallis analysis) as compared to 50 mg/kg. By contrast, no significant reduction in metastatic activity was observed at 50 mg/kg of ALP, and the greatest reduction in neoplastic foci was observed at 10 mg/kg.

DISCUSSION

Previous studies with ALP have shown that this class of compounds possesses antitumor properties in several experimental animal systems (29) and specific cytotoxic or cytostatic effects in vitro against animal and human tumor cells (30-32). Studies of the primary mode of action have focused on the augmentation of cytotoxic macrophages in vitro and in vivo (13,33). Most recently, Andreesen et al. (33) demonstrated augmentation of cytotoxic human macrophages.

The present study has shown that ALP had no NK cell-augmenting effects in vivo or in vitro. However, ALP significantly increased [^{3}H]thymidine incorporation into an allogenic MLR or into lymphocytes cultured alone, i.e., it demonstrated blastogenic activity. By contrast, ALP is unable to increase the development of cytotoxic effector cells following an in vitro allogenic MLTR-CMC assay. Nonetheless, ALP had significant adjuvant activity in vivo when administered with syngeneic tumor vaccines to generate cytotoxic T lymphocytes. However, this was a modest level of activity, and other positive controls such as muramyl dipeptide demonstrated greater activity (results not shown). In addition, ALP were able to activate macrophages both in vitro and in vivo. However, the ability to activate macrophages in vitro was obscured by the nonspecific adsorption of ALP. There was significant nonspecific adherence to the tissue culture plastic, resulting in cytotoxicity in the absence of macrophages when appropriate control studies were undertaken. In addition, when we used a control cell line rather than macrophages as effector cells, nonspecific adsorption of ALP was increased compared to the level observed in tissue culture wells containing ALP alone. Under these circumstances, we observed no difference in cytotoxicity in the presence or absence of macrophages. By contrast, the results of in vivo augmentation of peritoneal exudate macrophages with ALP were somewhat easier to understand. Under these conditions, significant tumoricidal activity was observed 24 hr following the i.p. injection of ALP. Nonetheless, it is still possible that ALP was membrane-associated and resulted in direct cytotoxicity rather than activation of the macrophages. This explanation is somewhat unlikely since the ALP was injected 24 hr prior to lavage of the peritoneal exudate cells, and the macrophages were allowed to attach for 2 hr and then were extensively washed to remove both nonadherent cells and any ALP that might have been membrane-associated. Studies by Andreesen et al. (33) have also addressed the specific adherence of ALP; these authors concluded that ALP could be adsorbed onto macrophage membranes in addition to directly activating the macrophages. In the present study, the therapeutic activity of ALP was observed in both experimental and spontaneous metastases. In the experimental metastasis model (Table 8), no therapeutic activity was seen when ALP were administered twice a week, although significant toxicity was observed at the highest dose. In contrast, in the spontaneous metastasis model (Table 9), significant therapeutic activity was observed, in the absence of overt toxicity, when ALP were injected three times a week. However, no toxicity was observed when ALP were administered at 50 mg/kg in this experiment, whereas significant toxicity was observed in the experimental model at 100 mg/kg of ALP (Table 8). This suggests that the therapeutic activity of ALP may be associated with a near-toxic dose. Another difference between the two models is that ALP was administered three times and two times per week in the spontaneous and experimental metastasis models, respectively (Tables 8 and 9). In other studies, therapeutic activity has been observed in experimental metastases, as determined by prolongation of survival at 5 and 15 mg/kg of ALP when it was administered three times per week.

The tendency of ALP to kill tumor cells at low concentrations and normal cells only at higher concentrations may result from metabolic differences between the two cell types. For example, synthetic ALP can be degraded by an enzyme that is present in normal cells; this substance is absent from tumor cells, leading to an accumulation of ALP in tumor tissues (8,34-36). Recent reports have suggested that incorporation of ALP into cytomembranes, with consequent disturbances of normal phospholipid metabolism, may result in cytostatic or cytotoxic properties (19). Further, other studies have shown that 1-alkyl glycerophosphatide analogs that activate macrophage cytotoxicity can inhibit enzymes involved in regulation of cytoplasmic arachidonic acid in murine macrophages (36).

In summary, ALP has some slight immunomodulatory and immunotherapeutic activities. Much of the in vitro macrophage activation associated with ALP appears to result from nonspecific adsorption of ALP rather than to "true" activation of macrophages. In addition, the therapeutic activity of ALP, while significant, is relatively minor and can only be achieved at nearly toxic doses. Thus, much of the therapeutic activity of ALP may relate to their direct toxic activity against tumor cells (8,34-37) rather than to their immunomodulatory properties.

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Neoplastic Cell Inhibition with New Ether Lipid Analogs[1]

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Bioactive phospholipid analogs of platelet-activating factor (PAF) represent a new approach to cancer chemotherapy. Various modifications of the basic structure of PAF lead to different ether lipid (EL) analogs. Data from the evaluation of thioalkyl and amidoalkyl glycerophosphocholine and of glycerophosphoinositol EL analogs against different experimental tumors in vitro (HL60 and K562 human leukemia cells, BG1 and BG3 ovarian adenocarcinomas) are presented. Exclusion of trypan blue after short exposure to the drugs determined cytotoxicity, and a soft agarose clonogenic assay measured the ability of the analogs to inhibit tumor cell proliferation. The thioalkyl EL are very active against the cell lines using both end points, and the amidoalkyl EL showed efficacy against the leukemic cell lines, whereas the phosphoinositol EL are active only at high concentrations. Combined use of EL analogs, which are membrane-interactive, with classical DNA-interactive chemotherapeutic drugs revealed that the combinations have additive antiproliferative effects. These results are promising leads in the development of the anticancer potential of ether lipid analogs.
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Bioactive phospholipid analogs of platelet-activating factor [1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, PAF] possess a broad range of biological activities and represent a new approach to cancer chemotherapy (1,2). These ether lipid (EL) analogs express their antitumor properties by direct cytostatic or cytotoxic action (1–3), by activation of macrophages (2–4) and by differentiation of malignant cells (5). They interact with the plasma membrane but not with DNA: they do not interfere with DNA synthesis or function and are not mutagenic (1). Definition of their mechanisms of antitumor action requires further research.

In view of this, we have initiated a comprehensive program on the design and synthesis of new EL analogs, together with parallel biochemical, biological and pharmacological studies and structure-activity correlations, aimed at identifying new active EL analogs of possible clinical interest. Some structure-antitumor activity studies have been done (6,7); this report updates the structure-activity data of our collaborative program. We have described preliminary results with 1-thioalkyl-2-alkylglycero-3-phosphocholine analogs (thioether GPC lipids) (8–11). This communication presents further data on the thioether lipid analogs and gives preliminary results of studies with two new classes of ether lipids: 1-amidoalkyl-2-alkylglycero-3-phosphocholine analogs (amidoether GPC lipids), reported for the first time, and 1,2-dialkylglycero-3-phosphoinositol analogs (GPI lipids), which have been described briefly before (12) (Fig. 1). In view of the fact that early clinical trials have already begun with two EL analogs (1,13,14), these new thioalkyl, amidoalkyl and GPI EL analogs are of particular interest. Also, to explore the potential of EL analogs as cancer chemotherapeutic agents, experiments were conducted to determine their antiproliferative activity in combination with conventional cytotoxic agents.

MATERIALS AND METHODS

All of the new EL analogs used in this study were synthesized at the University of North Carolina at Chapel Hill. Synthesis of the thioether lipid analogs has been described (9). Reports on the synthesis of the glycerophosphoinositol (GPI) and the amidoether lipid analogs are in preparation (12,15). ET-18-OMe was provided by Wolfgang Berdel, Technical University of Munich (Federal Republic of Germany); CP 46665 was a gift from Keith Jensen, Pfizer Co. (Groton, CT). Structures and abbreviations of the EL analogs studied are given in Figure 1. The structure of CP 46665 is given in ref. 20. The EL analogs were dissolved in ethanol with the exception of ET-18-OAc-GPI, for which chloroform was used. These stock solutions were diluted with phosphate saline buffer (PBS) prior to use; the final concentration of the organic solvent in the assay mixture did not exceed 0.5% for the viability test or 0.2% for the clonogenic assay.

Adriamycin was obtained from Adria Laboratories (Dublin, OH); Cisplatin was from Bristol-Myers (Wallingford, CT); and 4-hydroperoxycyclophosphamide (the active form of cyclophosphamide) was provided by O. Michael Colvin, Johns Hopkins Oncology Center (Baltimore, MD). Aliquots of these drugs were dissolved in PBS and cryopreserved under liquid nitrogen.

Cell lines of human leukemias HL60 and K562 (American Type Culture Collection, Bethesda, MD) were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml of penicillin and 100 μg/ml of streptomycin (all from Gibco, Grand Island, NY). Human ovarian carcinoma cell lines BG1 and BG3 (16) were propagated as monolayers in McCoy's 5A culture media (Gibco) with 10% fetal calf serum, 0.1% penicillin and 100 μg/ml of streptomycin. Serial passaging following mild trypsinization was done weekly in culture flasks (Costar, Cambridge, MA). Cells were free of mycoplasma contamination (17).

The biological activity of the drugs was evaluated using two different techniques. The cytotoxic potential of the compounds against HL60 and K562 was measured by a vital stain, trypan blue (18). Different concentrations of each drug were incubated with 5×10^5 cells/ml in 16-mm culture dishes. After 48 hr at 37 C in the presence of 5% CO_2 and 95% humidified air, viable cells were enumerated as those that retained a cell membrane permeability barrier against trypan blue dye intrusion. A hemocytometer was used to count the cells.

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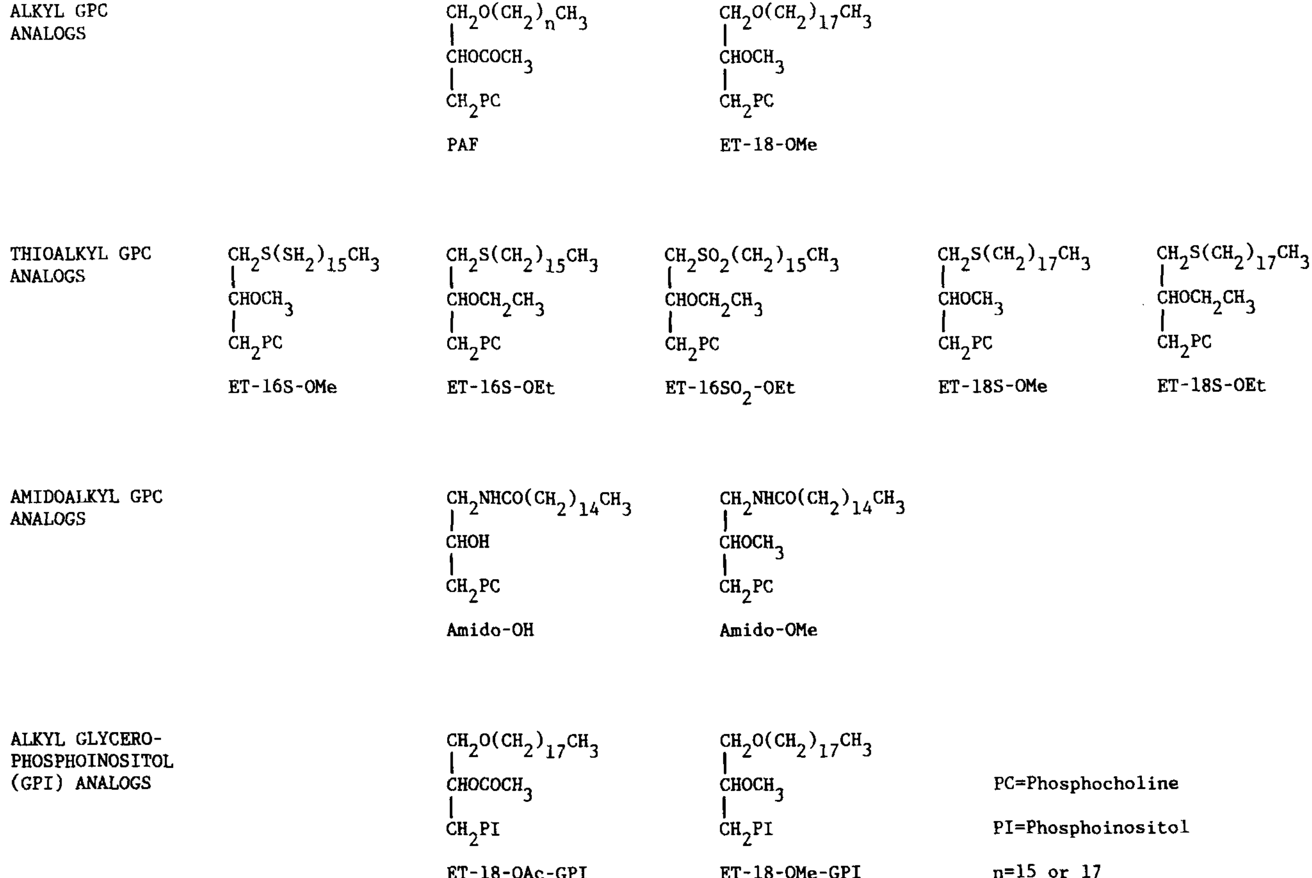


FIG. 1. Structures of PAF, 1-alkyl, 1-thioalkyl and 1-amidoalkyl glycerophosphocholine (GPC) ether lipid analogs and 1-alkyl glycerophosphoinositol (GPI) ether lipid analogs.

To determine changes in the clonogenicity of tumor cells resulting from treatment with drugs, a modified two-layer semisoft agarose assay was used (19). In brief, underlayers of 0.8% agarose in McCoy's media were prepared in 35-mm culture dishes and allowed to gel at 4 C. Single cell suspensions of the cell lines were prepared in McCoy's (BG1 and BG3) or in RPMI 1640 (HL60 and K562) with standard supplements and were mixed with molten agarose to achieve a final agarose concentration of 0.3%. One ml aliquots containing 3×10^4 ovarian carcinoma cells or 5×10^4 leukemic cells were applied to the underlayers and allowed to gel. Briefly laying the culture plates on a chilled marble slab ensured the formation of an even, firm gel. Drugs were diluted from reconstituted stock and applied one time to the two-layer agarose culture. Drugs were made as a 10× concentration of the desired final treatment in sterile PBS. Aliquots of 220 μl (1:10 dilution in the 2 ml agarose assay) were applied. (For the combination chemotherapy experiments, drugs were made as 20× concentration of the final treatment by inverted microscopy, and 110 μl aliquots of each drug were applied.) All agarose dishes were examined by inverted microscopy to ensure that cells were plated as a single-cell suspension. The cultures were incubated at 37 C and 7.5% CO_2 in humidified air for 7–10 days, during which time cell growth was monitored by inverted microscopy. Colony enumeration was done by automated image analysis (20). Colonies were defined as cell aggregates greater than 40μm in diameter. Each experiment was done in triplicate. Controls were run with a solution of 0.2 or 0.5% of solvent (ethanol or chloroform) in PBS.

RESULTS

Thioalkyl EL analogs. The thioalkyl analogs proved very effective against both leukemic cell lines in the cytotoxicity test in comparison with ET-18-OMe, which served as a reference compound (Table 1). When K562 cells were used, higher doses were required to detect some activity, because this cell line appears to be less sensitive than HL60 to the cytotoxic action of EL (21,22). However, some thio analogs were very effective against K562, particularly ET-16S-OEt. The clonogenic assay using HL60 leukemic cells confirmed, in general, what was found in the viability tests (data not shown). Although it is clear that in the cytotoxicity assays HL60 cells are more responsive to EL inhibition than K562 cells, preliminary data using K562 in the clonogenic assay did not show this difference in sensitivity between the two cell lines (data not shown). These data, however, need further confirmation.

The thioalkyl analogs were very active against the two ovarian adenocarcinomas (Table 2); in most cases they were significantly more active than ET-18-OMe. In

TABLE 1

Evaluation of the Cytotoxicity of Ether Lipid Analogs Against Human Leukemic Cells in a Cytotoxicity Assay

Experimental tumor	Compound	Dose (μM)							n
		1.25	2.5	5	10	20	40	80	
HL60	ET-18-OMe	73 ± 18	44 ± 15	18 ± 5	10 ± 3				18
	ET-16S-OMe	76 ± 11	38 ± 13	15 ± 5	9 ± 3				8
	ET-16S-OEt	60 ± 7	34 ± 5	14 ± 3	9 ± 1				8
	ET-18S-OMe	64 ± 9	41 ± 11	15 ± 1	10 ± 1				7
	ET-18S-OEt	51 ± 7	_21 ± 3_**	13 ± 1	10 ± 1				4
	Amido-OMe	64 ± 24	53 ± 14	20 ± 4	13 ± 5				9
	Amido-OH	89 ± 29*	81 ± 29**	72 ± 26**	64 ± 29**				9
	ET-18-OMe-GPI				87 ± 6**	94 ± 6	65 ± 10	38 ± 5	3
	ET-18-OAc-GPI				110 ± 10**	140 ± 7	79 ± 4	13 ± 4	3
K562	ET-18-OMe	106 ± 14	103 ± 14	76 ± 14	59 ± 7	48 ± 3	37 ± 6		8
	ET-16S-OMe			81 ± 7	68 ± 5**	55 ± 11	45 ± 11		6
	ET-16S-OEt			_56 ± 6_**	_48 ± 5_**	_42 ± 6_*	40 ± 8		5
	ET-18S-OMe			84 ± 3	65 ± 5	_38 ± 5_**	_24 ± 5_**		3
	ET-18S-OEt			88 ± 9	82 ± 9**	63 ± 1**	_28 ± 5_**		3
	Amido-OMe			82 ± 1	78 ± 2	61 ± 2	45 ± 4		3
	ET-18-OMe[a]			86 ± 12	59 ± 13	48 ± 15	24 ± 9		4

Each value (mean ± SD of n assays) represents cell survival as percentage of control; control values are 0.900 ± 0.082 × 10^6 (HL60) and 0.982 ± 0.192 × 10^6 (K562) cells/ml. Values in italics indicate greater activity than ET-18-OMe. *, $p < 0.05$ and **, $p < 0.01$ compared to ET-18-OMe.

[a]Separate reference control (ET-18-OMe) used with amido-OMe in the K562 system.

TABLE 2

Antiproliferative Effect of Ether Lipid Analogs Against Human Ovarian Tumor Cell Lines in a Semisoft Agarose Clonogenic Assay

Experimental tumor	Compound	Dose (μM)								n
		0.3	0.62	1.25	2.5	5	10	20	40	
BG1	ET-18-OMe	103 ± 4	96 ± 8	70 ± 2	9 ± 1	4 ± 2	2 ± 1			6
	ET-16S-OMe	103 ± 26	103 ± 16	70 ± 9	23 ± 5**	6 ± 1	5 ± 2*			3
	ET-16S-OEt	_74 ± 5_**	_67 ± 1_**	_18 ± 2_**	_2 ± 1_**	2 ± 0.4	1 ± 0.3			3
	ET-18S-OMe	_86 ± 6_**	_82 ± 5_**	_43 ± 3_**	_3 ± 1_**	2 ± 0.5	2 ± 1			3
	ET-18S-OEt	_74 ± 1_**	_52 ± 6_**	_3 ± 1_**	_1 ± 0.5_**	_0.5 ± 0.3_**	1 ± 0.3			3
	ET-18-OAc-GPI			86 ± 9**	83 ± 9**	51 ± 4**	54 ± 6**	59 ± 1	19 ± 15	3
BG3	ET-18-OMe	86 ± 2	83 ± 4	21 ± 1	4 ± 2	1 ± 0.2	0.4 ± 0.1			6
	ET-16S-OMe	94 ± 9	89 ± 5	95 ± 6**	96 ± 5**	95 ± 4**	39 ± 2**			3
	ET-16S-OEt	96 ± 11	81 ± 1	22 ± 3	2 ± 1	1 ± 0.1	1 ± 0.2			3
	ET-18S-OMe	99 ± 3**	81 ± 3	25 ± 6	_1 ± 0.1_*	_0.4 ± 0.1_*	0.4 ± 0.1			3
	ET-18S-OEt	91 ± 8	_64 ± 2_**	_3 ± 1_**	_1 ± 0.1_*	1 ± 0.1	1 ± 0.3			3
	ET-18-OMe-GPI			91 ± 2**	90 ± 9**	113 ± 1**	93 ± 8**	64 ± 2	47 ± 4	3
	ET-18-OAc-GPI			120 ± 3**	109 ± 11**	94 ± 8**	105 ± 3**	107 ± 8	49 ± 3	3

Each value (mean ± SD of n assays) represents colony survival as percentage of control; control values are 1549 ± 337 (BG1) and 1965 ± 236 (BG3) colonies/well. Colonies ⩾ 40 μm in diameter were counted. Values in italics indicate greater activity than ET-18-OMe. 3 × 10^4 Cells/well were plated for each tumor. *, $p < 0.05$ and **, $p < 0.01$ compared to ET-18-OMe.

this proliferative assay, both cell lines indicated that the 2-ethyl analog was more active than the 2-methyl.

The 1-sulfonyl analog is less active than ET-18-OMe and the thioalkyl analogs (Table 3). This is particularly evident with the BG1 tumor. Our ID_{50} value for ET-18-OMe against HL60 cells agrees with that published previously (23).

Amidoalkyl EL analogs. The 1-amido-2-methyl analog was as cytotoxic as the reference compound against the HL60 leukemic cell line, whereas the 2-unsubstituted analog was significantly less potent (Table 1). There was no statistical difference in inhibition of K562 cells by the 1-amido-2-methyl analog or by ET-18-OMe (Table 1).

GPI EL analogs. Synthetic modification of the EL analogs to introduce phosphoinositol instead of phosphocholine into the molecule (12) substantially reduced potency (Tables 1 and 2). Inhibitory activity was observed only at high concentrations, at levels considerably higher

TABLE 3

Comparison of ID_{50} of ET-16SO_2-OEt and ET-18-OMe in Two Different Assays

	ET-16SO_2-OEt ID_{50} (μM)	ET-18-OMe ID_{50} (μM)
HL60, cytotoxicity test	3.1 ± 0.5	2.5 ± 0.4
BG1, clonogenic assay	5.2 ± 0.1**	1.4 ± 0.1

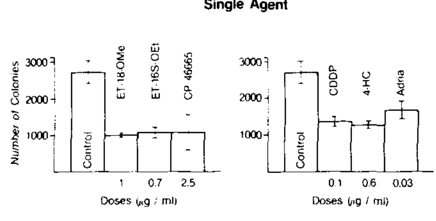
These values are the mean ± S.D. of 10 (HL60) and 3 (BG1) assays for ET-16SO_2-OEt and of 18 (HL60) and 6 (BG1) assays for ET-18-OMe. **, $p < 0.01$ compared to ET-18-OMe.

than the thioalkyl or dialkyl EL. The GPI EL analogs with either acetyl or methyl substitution at position 2 had similar degrees of inhibitory activity.

Combination chemotherapy. Figure 2 shows results of a combined administration of three different EL analogs with adriamycin, cisplatin and cyclophosphamide. The EL analogs were ET-18-OMe, ET-16S-OEt and CP 46665, a lipoidal amine for which antitumor activity has been previously described (24). These agents have different potencies in our system (8,10). Doses that inhibited ca. 50% of colony growth were used for all six drugs against the BG1 ovarian adenocarcinoma in the clonogenic assay.

The data obtained indicate that simultaneous use of these two different classes of agents provides enhanced inhibition of neoplastic cell growth. Statistical analysis of the data (16) revealed that the binary combinations of each EL analog with each DNA-interactive drug produced independent and additive effects and that neither synergism nor antagonism occurred. The thio analog had the strongest additive effect, followed by ET-18-OMe. Combinations of DNA-interactive drugs with CP 46665 showed weak additive activity.

Single Agent



Combination

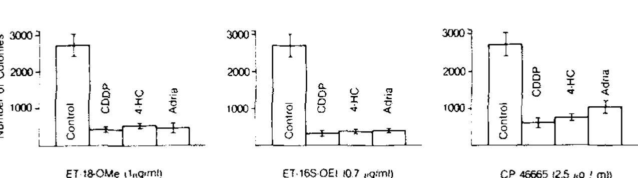


FIG. 2. Combination chemotherapy with DNA-interactive drugs: Adriamycin (Adria), Cisplatin (CDDP) and 4-hydroperoxycylophosphamide (4-HC) were used in a combined treatment with three ether lipid analogs in a semisoft agarose clonogenic assay against BG1 ovarian adenocarcinoma cells. The structure of CP46665 is given in ref. 20.

DISCUSSION

Thioalkyl EL analogs. The thioalkyl EL were designed to be more lipophilic compounds, which might therefore interact more readily with the cell membrane (8,9), the presumed lethal target for EL compounds (1). Three of these derivatives (ET-16S-OEt, ET-18S-OMe and ET-18S-OEt) were found to be very active against all the cell lines tested and in many cases significantly better than the reference compound, ET-18-OMe. ET-16S-OMe and the 1-sulfonyl analog showed cytotoxic activity comparable to that of ET-18-OMe only against HL60. Relative to the structure-activity relationship, our results indicate that (i) substitution of the ether linkage with thioether can in some cases increase the activity of EL analogs against human tumor cell lines; (ii) ethyl substitution at position 2 generally leads to more activity than methyl group substitution at this position; and (iii) the 1-sulfonyl derivative showed weaker activity than ET-18-OMe. This agrees with similar data recently reported (7).

Our data indicate that the thio analogs are one of the most active classes of EL analogs studied to date. After further preclinical studies, clinical use of one of these agents either alone or in combination with other antineoplastic agents may merit consideration. A thioalkyl EL very similar to our compounds (BM 41440) has been reported (25) to show promising antineoplastic activity in vitro and in vivo; after extensive preclinical evaluation, it is now in early clinical trials (14).

Amidoalkyl EL analogs. A 1-octadecyl-2-amido GPC analog has been described by Hoffman et al. (26) as having high activity against HL60 cells in vitro. Our approach was to assess the effect of substitution at position 2 on the activity of analogs with amido substitution at position 1. The results indicate that the 1-amido-2-methyl EL shows activity comparable to that of ET-18-OMe and superior to that of the 2-unsubstituted EL analog. Whether ethyl substitution at position 2 potentiates activity in amido-substituted EL analogs to the same degree as found in the thioalkyl-substituted compounds remains to be seen. This avenue of investigation is in progress.

GPI EL analogs. GPI analogs were designed on the basis that they might interfere with cellular proliferation by oncogene product inhibition at two points in the phosphatidylinositol cascade (8): (i) by interrupting phosphatidylinositol turnover at an early point in the cycle and (ii) by inhibition of protein kinase C, either directly or through inhibition of diacylglycerol formation, with resulting loss of cell-directed activation of the enzyme. Some activity was observed against experimental tumor cells in vitro (Tables 1 and 2). However, high doses are necessary to document this effect, and these analogs are much less active than ET-18-OMe or ET-16S-OEt.

Detailed biochemical studies are necessary to characterize the nature of the antiproliferative action of these phosphoinositol analogs. The results of the antitumor activity of these compounds are interesting because conclusions about structure-activity relationships can be made. Two GPI analogs of PAF and ET-18-OMe were tested. It has been demonstrated that PAF is far less active than ET-18-OMe as an inhibitory agent against cancer cells (8,26). PAF is readily deacetylated and thus metabolized to an inactive compound (27). This may

account for its diminished efficacy compared to other GPC analogs with methyl or ethyl substitution in position 2 (26–29).

Interestingly, the 2-acetyl GPI analog has the same activity as the 2-methoxy GPI analog; in other words, the difference in inhibitory activity so clearly evident with PC substitution at position 3 is not observed with PI substitution in the same set of analogs.

All these data are derived from in vitro studies. In vivo experimentation is now being carried out with transplantable mouse tumors to determine the therapeutic efficacy of the three groups of compounds. Normal mice and rats are being used to characterize the toxicology, metabolism and pharmacokinetics of these analogs. Also athymic mice bearing subcutaneous BG1 tumors will be treated with the different analogs; this should permit the comparison of in vitro and in vivo data directly with the same human malignant cells.

Combination chemotherapy. A most interesting and unusual feature of the EL analogs is that they act through a membrane interaction without affecting the DNA (1). Even though the precise molecular mechanism by which the antiproliferative action is exerted is still unknown, this drug-membrane relationship is well established (30–32). We have recently reported that the EL can easily interact with model membranes altering their physical properties (33). Thus it seemed reasonable to evaluate these drugs in combination with agents with a different mechanism of action to determine if this would lead to a potentially effective new approach to chemotherapeutic treatment. Additive interactions were observed for combinations of three different EL with DNA-interactive drugs (Fig. 2). This suggests that the two classes of agents operate via independent mechanisms in expressing their antiproliferative effects. ET-18-OMe has been reported to exhibit synergistic inhibition of human leukemic cells in vitro in combination with vincristine (34).

This preliminary finding is important from two points of view. First, these data indirectly confirm that the membrane is a specific target for the EL analogs; a synergistic inhibition might be expected if the EL analog enhanced the uptake of the DNA-interactive agent, as observed for vinblastine uptake in KB cells in the presence of lysophosphatidylcholine or phosphatidylinositol (35). Second, further preclinical investigation may be warranted, as this combination chemotherapy approach may provide a means of modifying the toxicity of both drugs while increasing their therapeutic efficacy.

Our results suggest three significant points in the development of EL analogs for antitumor therapy: (i) thio substitution at position 1 potentiates cytotoxic and antiproliferative activity of these compounds; (ii) a 1-amido analog is as cytotoxic as ET-18-OMe; and (iii) in combination with DNA-interactive drugs being used, EL show additive antitumor effects. Substitution of sites on the EL backbone with the goal of optimizing activity is under active investigation by our institutions as a promising approach to advancing this new class of anticancer compounds.

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Antitumor Activity of SRI 62-834, A Cyclic Ether Analog of ET-18-OCH_3[1]

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SRI 62-834, an analog of the antitumor agent ET-18-OCH_3 in which the oxygen atom at carbon atom 2 has been incorporated into a five-membered heterocycle, has been prepared and evaluated as an antitumor agent. The compound exhibited good cytotoxicity in vitro against a variety of tumor cell lines and was as effective as ET-18-OCH_3 given orally in the mouse Meth A sarcoma model. SRI 62-834 was shown to be an inhibitor of platelet-derived growth factor (PDGF), possibly at the receptor level, and platelet-activating factor (PAF) at the receptor level.

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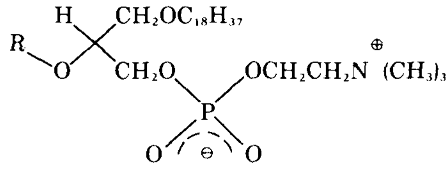

FIG. 1. Structure of ET-18-OCH_3 (R = CH_3) and platelet-activating factor (R = $COCH_3$).

The ether phospholipid ET-18-OCH_3 (Fig. 1) has been shown to exhibit neoplastic activity in vitro and in vivo against a wide variety of tumor cell lines (1). Considerable preclinical studies suggest that the antitumor action of this agent may be attributed to generation of cytotoxic macrophages (2–4), direct cytotoxicity (5), diminished activity of the alkyl cleavage enzymes present in tumors (6–9), selective cell membrane interactions (10) and inhibition of a phospholipid cofactor of a phospholipid-sensitive Ca^{++}-dependent protein kinase (11). In addition, it has been shown that ET-18-OCH_3 can purge murine leukemic bone marrow, eliminate leukemic blasts and allow hematopoietic reconstitution to occur (12). Unlike most substances being used in the clinical treatment of tumors, ET-18-OCH_3 does not appear to have a direct effect on DNA synthesis or functions, and it is nonmutagenic (5,13). Clinical phase I and II studies with ET-18-OCH_3 against a variety of advanced solid tumors have recently appeared (1,14).

Our laboratories have been involved in the synthesis and evaluation of a variety of cyclic ether analogs of ET-18-OCH_3 as potential antitumor agents (Houlihan, W.J., Munder, P.G., Lee, M.L., Parrino, V.A., and Cheon, H., unpublished data). Here we present our findings with SRI 62-834, a tetrahydrofuran analog of ET-18-OCH_3 (cf Fig. 2), against a variety of tumor cell lines, platelet-derived growth factor (PDGF) and platelet-activating factor (PAF).

MATERIALS AND METHODS

Chemical synthesis. Melting points were determined on a Thomas Hoover melting point apparatus and are uncorrected. Nuclear magnetic resonance (NMR) data for ^1H-NMR were taken on JEOL-FX-90 (90 MHz) or JEOL-FX-200 (200 MHz) spectrophotometers and are reported in δ (ppm) downfield from tetramethylsilane (TMS). ^{13}C-NMR were determined at 22.5 MHz or 50.1 MHz on the JEOL instruments, respectively, with $CDCl_3$ (77.0) and TMS (0.0) as internal reference. ^{31}P-NMR was measured at 80.76 MHz on a JEOL-FX-200 with H_3PO_4 as external reference. If not otherwise specified, chemicals and reagents were obtained from the Aldrich Chemical Co. (Milwaukee, Wisconsin). Solvents were of reagent grade and dried prior to use. Reaction progress and purity of final products were determined on E. Merck Silica Gel 60 chromatography plates. Column chromatography was carried out using E. Merck Silica Gel CH83 (0.06–0.20 mesh) with the indicated eluants. Eluants and Rf values are reported where appropriate. The purity of SRI 62-834 was also assessed by high pressure liquid chromatography (HPLC) at 1500 psi on a Beckman 345 instrument with RI detection using a Lichrosorb Si 60 5 μ dp column eluted with methanol/chloroform/water (6:5:0.5, v/v/v) at 1.5 ml/min. Retention time is reported at a chart speed of 0.2 cm/min.

(±)Methyltetrahydro-2-furoate. A suspension of 2-furoic acid (34.9 g, 0.31 mol), 95% ethanol (125 ml) and 10% palladium on carbon catalyst (1.6 g) in a pressure bottle was affixed to a Parr hydrogenation apparatus under 45 psi hydrogen and maintained at room temperature. After hydrogen uptake was complete (ca. 5 hr), the reaction mixture was filtered through a bed of celite at room temperature. The filter bed was washed with 95% ethanol, and the combined filtrates were concentrated in vacuo to yield 37.7 g of crude tetrahydro-2-furoic acid. The acid (37.7 g) was dissolved in a saturated hydrogen chloride–methanol solution (200 ml) and stirred at room temperature for ca. 5 hr. The solvent was removed in vacuo, and the residue was taken up in methylene chloride (150 ml), washed with 10% sodium bicarbonate (twice 150 ml) and water (twice 150 ml), dried with anhydrous magnesium sulfate, filtered and concentrated in vacuo to give 30.0 g (77%) of a colorless oil. Anal. calc. C, 55.4; H, 7.7. Found C, 55.2; H, 7.8.

2,2-bis-Hydroxymethyltetrahydrofuran. A stirred solution of methyltetrahydro-2-furoate (21.0 g, 0.16 mol) in anhydrous tetrahydrofuran (180 ml) under a nitrogen atmosphere was cooled to an internal temperature of −65 ±5 C and then treated dropwise with 25% diisobutylaluminum hydride solution in toluene (102 g, 0.17 mol) at such a rate that the internal temperature did not exceed −60 C. The mixture was stirred for an additional 1.5 hr at −60 C and then treated carefully with anhydr-

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ous methanol (23 ml) such that the temperature did not exceed −50 C. The solution was allowed to warm to 5 C and then was added to a stirred solution of 37% aqueous formaldehyde (226 ml, 3.9 mol), sodium hydroxide (52 g, 1.3 mol) and water (170 ml) while maintaining the internal temperature at 10 ± 5 C. The mixture was treated with formic acid (6.8 ml) and then concentrated in vacuo with a water aspirator at 65 ± 5 C until distillation ceased. The residue was cooled to 25 C, treated with methylene chloride (90 ml) and stirred for ca. 0.25 hr. The resultant slurry was filtered, and the solid washed with methylene chloride (360 ml). The filtrate was concentrated in vacuo on a rotary evaporator, and the residue distilled (bp 108–120 C at 0.2–0.3 mm Hg) to give a semisolid that crystallized from toluene to give 10.5 g (50%) of a white solid; mp 58–60 C (lit. [15] mp 51–53 C).

(±)-2-Hydroxymethyl-2-octadecyloxymethyltetrahydrofuran (9.9 g, 0.075 mol) in dimethyl sulfoxide and tetrahydrofuran. To a stirred solution of 2,2-bis-hydroxymethyltetrahydrofuran (1:1, 100 ml) was added at room temperature powdered potassium hydroxide (4.2 g, 0.075 mol) followed by dropwise addition of n-octadecylbromide (8.4 g, 0.025 mol) in tetrahydrofuran (30 ml). The reaction mixture was stirred for ca. 16 hr and concentrated in vacuo. The residue was treated with water (100 ml) and extracted with diethyl ether (100 ml, three times). The ether layer was washed with saturated sodium chloride solution, dried with anhydrous magnesium sulfate, filtered and concentrated in vacuo. The residue (11.0 g) was chromatographed (1:1 methyl t-butylether/hexane followed by 3:1 methyl t-butylether/hexane) to give 5.8 g (33%) of a white sticky solid, Rf 0.52 (methyl t-butylether), MS (isobutane DCI) mz 356 (MH^+). Anal: Calc C, 74.1; H, 12.4. Found: C, 74.0; H, 12.5.

(±)-2-{Hydroxy[tetrahydro-2-(octadecyloxy)methylfuran-2-yl]methoxyl]phosphinyloxy}-N,N,N-trimethylethaniminium hydroxide, inner salt (SRI 62-834). To a stirred solution of (±)-2-hydroxymethyl-2-octadecyloxymethyltetrahydrofuran (2.9 g, 7.6 mmol) in anhydrous benzene (50 ml) maintained under a nitrogen atmosphere was added at room temperature anhydrous triethylamine (1.22 ml, 8.8 mmol), 4-dimethylaminopyridine (86 mg) and 2-chloro-2-oxo-1,2,3-dioxophospholane (16) (1.4 g, 9.8 mmol). The reaction mixture was stirred for 16 hr at room temperature and then concentrated in vacuo. The residue was dissolved in anhydrous acetonitrile (40 ml) and placed in a pressure bottle equipped with a magnetic stirring bar. The bottle was cooled in a dry ice acetone bath and anhydrous trimethylamine gas was bubbled in for ca. 15 min. The bottle was then capped and heated at 60–65 C for 48 hr. After being cooled to ca. −20 C (ice-methanol), the solid was filtered off, washed with acetonitrile (5 ml) and chromatographed (chloroform/methanol/water; 70:35:7, v/v/v) to give a white solid. The solid was dissolved in ethanol (15 ml, three times) and evaporated to remove traces of water. Crystallization from chloroform/acetone (1:1, v/v) (15 ml) gave 2.1 g (49%) of a white solid mp > 230 C; Rf 0.3 ($CHCl_3/CH_3OH/H_2O$; 70:35:7, v/v/v); HPLC retention time 7.22 min.; ^1H-NMR ($CDCl_3/CD_3OD$) δ 0.88 (t, 3H, J = 7.5, CH_3) 1.25 (s, 30 H) 1.51 (m, 2H), 1.91 (m, 3H), 3.21 [s, 9H, $^+N(CH_3)_3$], 3.37 (s, 2H), 3.41 (d, 2H), 3.58 (brs, 2H), 3.82 (m, 4H), 4.25 (brs, 2H); ^{13}C-NMR ($CDCl_3/CD_3OD$) ppm 29.436 and 29.582 [$(CH_2)_n$], 54.254 [$N^+(CH_3)_3$] 67.715 (CH_2N^+), 67.931, 68.707, 71.890, 73.350 (various CH_2O); ^{31}P-NMR ($CDCl_3/CD_3OD$) 1.22 pp.; MS (isobutane DCI) m/z (rel int.) 550 (M^+, 15), 367 (M^+-OPC, 100). Anal. for $C_{29}H_{60}NO_6P{\cdot}H_2O$: Calc. C, 61.3; H, 10.9; H, 2.4. Found: C, 61.4; H, 11.1; N, 2.3.

Cell lines. The tumor cell line Abelson-8.1 was obtained from A.W. Harris (Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) and the YAC-1 from G. Klein (Department of Tumor Biology, Karolinska Institute, Stockholm, Sweden). The cells were grown in stationary suspension culture in Dulbecco Modified Eagle's Medium (DMEM) and 10% heat inactivated fetal calf serum (FCS) supplemented with 50 μM 2-mercaptoethanol, 100 units penicillin and 1 μg streptomycin. The P815 and L1210 cells were from the Max-Planck-Institut fuer Immunbiologie cell line collection and were passed intraperitoneally (ip) in vivo every week. The MethA fibrosarcoma cells were originally induced in BALB/C mice by administering methylcholanthrene according to the procedure of Old et al. (17).

The human neuroblastoma cells were obtained by dissociating and culturing a freshly explanted tumor specimen. The cells were used after weekly passage for four weeks in DMEM and supplements.

Human foreskin fibroblasts (AG1523) were obtained from the Institute of Medical Research (Camden, New Jersey) and propagated from frozen stock in DMEM (pH 7.8) supplemented with 20% FCS, 1% nonessential amino acids (Gibco Cat. No. 320-1140), 1% essential amino acids (Gibco Cat. No. 320-1135), 1% vitamins (Gibco Cat. No. 320-1120), 6 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. The cells (passages 6 or 7) were maintained at 37 C under a humidified atmosphere of 95% air/5% CO_2.

Macrophages were grown from the bone marrow cells of (Balb/C × $C57/bl_6)F_1$ mice as described elsewhere (8). Cultivation was effected by placing a suspension of 3 × 10^6 bone marrow cells/50 ml, DMEM, 10% FCS, 5% horse serum and 3% supernatant of L929 fibroblast culture containing stimulating factor into ethylene oxide sterilized 30 × 5 cm Teflon bags (Biofolie, Heraeus, Hanau, FRG) as previously described (18). The cells are cultivated on the hydrophobic side of polymeric fluorocarbon film, and in 8–12 days the precursor cells develop into mature macrophages (19). Macrophages are brought into single cell by rolling the cultivation suspension side of the bag slightly without pressure between two fingertips. After 12 min, the moderately attached macrophages came off. After sterilizing, the cell suspension was centrifuged on a Ficoll hypaque layer at 500 g for 30 min, and the mononuclear cells were washed, first at 400 g for 10 min and then at 250 g for 10 min.

Biochemicals and radiochemicals. ET-18-OCH_3 (rac-1-octadecyl-2-methoxy-3-phosphorylcholine) was obtained from R. Berchthold (Biochemicals Laboratory, Bern, Switzerland); human PDGF (300,000–500,000 units/mg where one unit is defined as the amount of PDGF needed to stimulate the incorporation of [^3H]thymidine into 3T3 fibroblast DNA to the same extent as 5% calf serum [20]) was obtained from Cellular Products, Buffalo, NY; [^3H]thymidine was obtained from Amersham and Buchler (Braunschweig, FRG) or ICN (Irvine, CA); ^{125}I-PDGF was prepared by Dr. Larry D. Witte (Columbia University, College of Physicians and Surgeons, New York City, NY) using the method outlined by Bowen-Pope and Ross (21); ^3H-PAF (9,10-1-O-octadecyl-^3H) was prepared in the

laboratories of the Sandoz Research Institute by the procedure of Morgat (22).

Tumor cell cytocoxicity. Abelson-8.1 tumor cells (1×10^3 cells/well) in DMEM and 10% FCS were placed in flat-bottom microtiter plastic plates (Nunc Roskieide, Denmark) and incubated with 5 μg/ml of SRI 62-834 or ET-18-OCH_3 dissolved in water for 24–72 hr. The number of tumor cells present was determined by measuring alkaline phosphatase activity by a modified procedure of Culvenor (23); the tumor cell plates were centrifuged at 500 $\times$g for 10 min and the supernatant was flicked off. Without further washing, 100 μl of buffer containing 20 μl of diethanolamine, 2 μM magnesium chloride hexahydrate, 2.5 μM p-nitrophenylphosphate and 10 mg Triton X-100 was added. The samples were incubated for 60 min at room temperature, and the enzymatic activity was terminated by the addition of 100 μl of 0.5 N sodium hydroxide. The absorbance was then measured at 405 nM using a Titertek Multiskan apparatus and compared to non-drug-treated cells. The same assay procedure was used to determine P815, YAC-1 and L1210 tumor cells.

The tumor cell growth of the MethA fibrosarcoma and human neuroblastoma were assayed by the procedure of Berdel et al. (4). After the incubation period, 0.5 μCi of [6-^{3}H]thymidine was added to each well, and allowed to incubate for 24 hr. The cells were collected with a multiple automated sample harvester (Mash II, Flow Laboratories, Bonn, FRG) on glass filter paper. The filters were dried at 55 C, and then transferred to scintillation vials containing 4 ml scintillation fluid (Scintigel, Roth KG, Karlsruhe, FRG). The radioactivity was measured in a liquid scintillation counter.

Macrophage cytotoxicity. Mouse macrophages (10^5/well) were incubated with 10 μg/ml of ET-18-OCH_3 for 24 hr in flat-bottom microtiter plates, after which they were centrifuged and washed once. Abelson-8.1 tumor cells (1×10^3/well) in DMEM and 10% FCS plus 5 μg/ml of SRI 62-834 or ET-18-OCH_3 were added to the plates and incubated for 72 hr. With the cytotoxicity of ET-18-OCH_3 (10 μg/ml) alone set at 100%, the enhancement of the cytotoxic effect was assayed by the [^{3}H] thymidine method desribed above.

Mouse MethA fibrosarcoma assay. Ten CBF_1 mice, 10–12 wk of age, were implanted with 10^5 MethA sarcoma cells to serve as control. Ten other CBF_1 mice were implanted with 10^5 MethA sarcoma cells and on day 1 after implant were each treated per os (p.o.) with 50 μg of SRI 62-834 or ET-18-OCH_3; daily drug treatment continued for 27 days. On days 7, 15, 21 and 28 after tumor implant, the entire tumor volume was calculated by the equation $V = 2/3\pi AB(AB + B/2)$, where A and B are measured tumor diameters.

Human fibroblast platelet–derived growth factor (PDGF) mitogen assay. Confluent fibroblast cultures in 96-well plates were quiesced at 37 C in growth medium containing 0.5% calf serum for 28 hr. Compounds to be tested or the appropriate vehicles were added to fibroblasts 1 hr before. The cultures were exposed to PDGF for 15 hr before [^{3}H]thymidine (2.0 μCi/ml) was included in the quiescing medium. Two hr after the addition of [^{3}H]thymidine, the medium was aspirated, and the fibroblasts were washed with Dulbecco's phosphate-buffered saline (ph 7.4) containing 1mM $CaCl_2$ and 1 mM $MgCl_2$ followed by 10% trichloroacetic acid and ethanol/ether (2:1, v/v). Phase-contrast microscopy was used to inspect the microwells for evidence of cell detachment or changes in cell morphology. Acid-insoluble [^{3}H]thymidine was extracted into 0.5 N NaOH, and the radioactivity was quantified with a gamma counter.

[^{3}H]Thymidine uptake. Human foreskin fibroblasts were propagated in 24-well plates (1×10^5 cells/well) and quiesced as described above for estimates of [^{3}H]thymidine incorporation into DNA. SRI 62-834 or its vehicle was added to the quiescing medium 1 hr before the addition of media. Fibroblasts were incubated with 5% calf serum for 15 hr and [^{3}H]thymidine (0.5 μCi/ml) was added. Thirty or 90 min after the addition of [^{3}H]thymidine, the uptake experiments were terminated by aspirating the media and washing the cell sheets twice with cold (0 C) Dulbecco's phosphate-buffered saline (pH 7.4) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. [^{3}H]Thymidine was extracted into 10% trichloroacetic acid at 20 C for 15 min. Aliquots of the acid extracts were neutralized with 0.5 N NaOH. Radioactivity in the neutralized extracts was quantified with a liquid scintillation counter.

^{125}I-PDGF specific binding. The specific binding of ^{125}I-PDGF to human foreskin fibroblasts was determined essentially as proposed by Bowen-Pope and Ross (21). Human foreskin fibroblasts which had been seeded into 24-well plates (5×10^4 cells/well) were incubated at 37 C for 96 hr in 1 ml of growth medium containing 20% FCS. The growth medium was not changed during this time. At the end of 96 hr, the growth medium was aspirated and the confluent cultures were washed twice with binding medium, which consisted of phosphate-buffered saline (pH 7.4) containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 mg/ml bovine serum albumin and 15 μg/ml phenol red. Fibroblasts were incubated with SRI 62-834 or its vehicle for 1 hr at 37 C, before ^{125}I-PDGF (6–11 ng/ml, 10-20 μCi/μg) was added to the cultures in the appropriate vehicle to a total volume of 350 μl. The incubation was continued for 2 hr at 4 C with gentle shaking. Nonspecific binding of ^{125}I-PDGF (20–40% of total binding) was estimated in the presence of a 50-fold molar excess of unlabeled PDGF. Binding assays were terminated by aspirating the radioactive medium and washing the cell sheets three times with 1 ml of fresh binding medium at 0 C. After the cultures had been inspected with a phase-contrast microscope for morphological changes, the cells were solubilized in 1% Triton X-100 containing 1 mg/ml bovine serum albumin by incubation at 37 C for 30 min. Radioactivity in aliquots of Triton-solubilized cells was quantified with a gamma counter.

Protein kinase C assay. Partially purified protein kinase C was prepared by a modification of the method described by Sahyoun et al. (24). Brains from male Sprague-Dawley rats (175–200 g) were homogenized with a Brinkman Polytron in ice-cold 50 mM Tris/HCl (ph 7.4 at 25 C) containing 10 mM dithiothreitol, 5 mM EGTA and 2 mM EDTA. The homogenate was incubated in the ice for 1 hr and then centrifuged at 100,000 $\times$ g for 30 min. The supernatant was passed through a PD 10 Sephadex G-25 M column and purified on a Pharmacia FPLC Mono Q anion-exchange column using a 0.05 M NaCl gradient containing 50 mM Tris-HCl (pH 7.4 at 25 C), 1 mM dithiothreitol.

Protein kinase C activity was determined by incorporation of ^{32}P into histone from [^{32}P]ATP by a modification of the method described by Castagna et al. (25). The assay

contained 10 μM [γ-^{32}P]ATP (0.5–1.0 μg/mM), 200 μg histone (Sigma, Type III), 5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.8 μg diolein, 40 μg phosphatidylserine and purified protein kinase C in 1 ml of 0.05 M Tris/HCl buffer (pH 7.4 at 25 C) together with 0.15–9.00 mM SRI 62-834. The assay was incubated at room temperature for 5 min and terminated by the addition of 1 ml of 25% trichloroacetic acid, and the assay tubes were filtered through Whatman G/F glass fiber filters. The filter papers containing the precipitated histone and incorporated ^{32}P were counted in a Beckman LS 8000 scintillation counter. Blank values were determined by assays run without phosphatidylserine and diolein.

Drug inhibition is expressed as the decrease in phosphatidylserine- and diolein-stimulated ^{32}P incorporation into histone.

Platelet aggregation inhibition. Human subjects were kept aspirin-free for one week and fasted overnight. Platelet-rich plasma (PRP) was prepared by centrifugation (250 × g, 10 min, 24 C) of freshly drawn blood anticoagulated with 0.38% sodium citrate (final concentration). Platelet count was adjusted to 2.5 × 10^5 per μl using platelet-poor plasma (PPP) obtained by a second centrifugation (900 × g, 15 min, 24 C) of the blood sample. An aliquot (0.38 ml) of the PRP was dispensed into cuvettes, incubated at 37 C and stirred at 900 rpm within a Payton Aggregometer, which recorded the light transmission pattern of the platelet suspension. The test compound was then added at final concentrations in the range of 0.1–100 μM. One minute after the addition of test compound, PAF-18 was added to the cuvette in an amount predetermined to give a consistent increase in light transmission (4.8 × 10^{-7} M). All aggregations were allowed to proceed for 5 min from the addition of the PAF. The aggregation response was quantitated by determining the area under the curve (AUC) using a plane polarimeter. The percent inhibition of the aggregation response was determined by dividing the AUC generated in the presence of the compound by the AUC of the PAF alone, multiplying by 100 and subtracting from 100.

Inhibition of [^{3}H]PAF binding to the human platelet PAF receptor. The binding assay is a modification of the procedure of Valone (26) and Kloprorogge (27) and the preparation of platelets a modification of the method of Radomski and Moncada (28). Freshly drawn human blood was anticoagulated with 0.38% sodium citrate and 2.8 μg/ml PGI_2. PRP was prepared by centrifugation of the blood at 250 × g for 10 min at 24 C. The platelets were sedimented by centrifugation at 900 × g for 15 min at 24 C and then washed twice with a solution of Tris-Tyrode's buffer (pH 7.4) containing 0.25% bovine serum albumin (TT/BSA) and 0.3 μg/ml PGI_2. The washed platelets were resuspended to 3.5 × 10^5 per μl in TT/BSA. Aliquots of platelets (500 μl) were mixed with 1.5 nM [^{3}H]PAF (40,000 cpm; 49 Ci/mmol). Nonspecific binding was estimated in duplicate tubes containing excess cold PAF (3.7 × 10^{-7} final concentration). After incubation at 24 C for 1 hr, the reaction was stopped by adding 500 μl ice-cold TT/BSA. The [^{3}H]PAF bound to platelets was separated from the free radioligand by centrifugation at 900 × g for 10 min at 4 C. Pellets were washed with TT/BSA and centrifuged at 900 × g for 15 min at 4 C. The pellets were resuspended in 2.5 ml liquid scintillation fluid, and the tubes were mixed and counted for one minute in a liquid scintillation spectrometer. The amount of specific binding was calculated as the difference in cpm between the total bound [^{3}H]PAF and nonspecifically bound [^{3}H]PAF (bound in the presence of 3.7 × 10^{-7} M cold PAF). The percent inhibition of specific binding was determined by dividing the cpm specifically bound in the presence of the test compound by the cpm specifically bound in the vehicle control, multiplying by 100 and subtracting from 100. An IC_{50} (50% inhibitor concentration) value was generated by evaluating the test compound over the concentration range of 0.1–100 μM.

PAF-induced guinea pig hemoconcentration (HC) and bronchoconstriction (BC). The methodology of Handley (29,30) for evaluation of PAF-induced HC in the guinea pig was used. Briefly, 100 ng/kg^{-1} PAF-18 was given intravenously, and blood samples were taken from the femoral artery for determination of hematocrit changes. For BC studies, animals were similarly prepared, tracheotomized and given flaxedil (gallamine triethiodide, American Cyanamid, Pearl River, NY; 10 mg/kg^{-1} ip) to suppress natural breathing. Animals were artificially ventilated (Harvard Respirator, 60 breaths min^{-1}, tidal volume of 3.5 ml/kg^{-1}), and bronchial resistance was determined from initial values, monitored with pressure transducers and a Gould 2400S recorder. SRI 62-834 in 0.9% saline (three animals per dose) was given intraarterially (ia) 5 min before the PAF challenge. Blood samples and bronchial tracings were taken during the predose time to determine if SRI 62-834 influenced these parameters.

CO_2H —a,b→ CO_2CH_3 —c,d→ CH_2OH / CH_2OH —e→ $CH_2OC_{18}H_{37}$ / CH_2OH —f,g→ $CH_2OC_{18}H_{37}$ / CH_2O–P(O)(O$^{\ominus}$)–$OCH_2CH_2N^{\oplus}(CH_3)_3$

SRI 62-834

(a) 5% Pd/C, H_2, 95% EtOH
(b) CH_3OH, HCl, r.t.
(c) $(i\text{-}Bu)_2AlH_2$, −60 C, THF
(d) CH_2O, KOH
(e) n-$C_{18}H_{37}Br$, KOH, DMSO-THF
(f) 2-chloro-2-oxo-1,3,2-dioxaphospholane (O, O, P, O, Cl), Et_3N, DMAP, C_6H_6, 2.5 hr, r.t.
(g) $(CH_3)_3N$, 60-65 C, 48 hr.

FIG. 2. Synthesis of SRI 62-834.

TABLE 1

Comparison of the In Vitro Effects of SRI 62-834 and ET-18-OCH_3 at 5 μg/ml on Various Tumor Cells

Tumor cells	Assay[b]	% Viable cells at 72 hr[a]	
		SRI 62-834	ET-18-OCH_3
Abelson-8.1[c]	DC	2.0	2.8
Abelson-8.1[c]	MAC	98.0[e]	97.8[e]
YAC[c]	DC	4.0[e]	4.3
L1210[c]	DC	5.8	5.3
P815[c]	DC	9.1	5.8
Human neuroblastoma[d]	DC	0.6	0.6
MethA fibrosarcoma[d]	DC	0.8	1.1

[a]Values are averages of quadruplicate assays and have an average error of ±4.2%.
[b]DC, direct cytotoxicity; MAC, macrophage-activated cytotoxicity.
[c]Viable cells measured by alkaline phosphatase activity.
[d]Viable cells measured by ^{3}H-thymidine uptake.
[e]Value is % enhancement.

TABLE 2

Comparison of SRI 62-834 and ET-18-OCH_3 in the Mouse MethA Fibrosarcoma Assay

Compound	Dose per os, μg/mouse	Tumor size, % control[a]				Survivors at day 28
		Day 7	Day 14	Day 21	Day 28	
Control	–	100	100	100	100	0/10
SRI 62-834	50	67	23	8	13	6/10
	5	82	29	14	19	4/10
ET-18-OCH_3	50	86	27	12	18	5/10
	5	81	28	10	16	6/10

[a]Values are averages of all surviving animals and have an average error of ±10.5%.

TABLE 3

Effect of SRI 62-834 on [^{3}H]Thymidine Uptake in Human Foreskin Fibroblast Cells

Compound	μg/ml	[^{3}H]Thymidine incorporation[a] (cpm)
5% Fetal calf serum	–	965 ± 38
SRI 62-834	2.5	1138 ± 284
	5.0	1022 ± 225

[a]Values are means ± SEM of three different culture assays.

TABLE 4

Effect of SRI 62-834 on Protein Kinase C Activity

Concentration (mM)	Enzyme activity[a] (% control)
0	100
0.15	86
0.30	96
0.90	71
1.80	31
5.40	25
9.00	21

[a]The control enzyme activity was 0.059 μ mol/mg/min.

RESULTS AND DISCUSSION

The synthesis of SRI 62-834 starting from the commercially available 2-furoic acid was accomplished in the seven synthetic steps given in Figure 2.

A summary of the direct cytotoxicity effects of SRI 62-834 and ET-18-OCH_3 on a variety of tumor cell lines is given in Table 1. The proliferation rate of the cells during a 72-hr period was reduced significantly when SRI 62-934 was included in the media at 5 μg/ml. Incubation of SRI 62-834 with preactivated mouse macrophages and Abelson-8.1 lymphoma cells for 72 hr showed the compound to be a highly effective macrophage activator resulting in ~ 98% elimination of the tumor cell inoculum (cf Table 1).

Evaluation of SRI 62-834 at ca. 2.5 mg/kg and 0.25 mg/kg, respectively, in the mouse MethA sarcoma model over a 28-day period showed that the compound had a very positive effect in controlling the size of tumor growth and increasing the number of survivors. The efficacy of SRI 62-834 compared to ET-18-OCH_3 appears to parallel the in vitro antitumor findings since both compounds have similar po activity in the mouse MethA sarcoma model (cf Table 2).

To determine whether SRI 62-834 had any effect on normal cell function in the concentration range that was toxic to tumor cells, it was incubated with human foreskin fibroblasts at 2.5 μg and 5.0 μg/ml, respectively, for a 15-hr period. The cells were then challenged with [^{3}H]thymidine, and 90 min later uptake was measured. At both doses, SRI 62-834 did not cause any alteration of uptake relative to the untreated control cells (cf Table 3). Examination of the cells after drug treatment by phase-contrast microscopy failed to uncover any morphological changes.

In recent years, a number of experiments have provided a connection between oncogenes and human cancer (31). One of the peptide chains of human PDGF, a cationic glycoprotein composed of two disulfide-linked peptide chains, shows amino acid sequence homology with the predicted protein products of viral *sis* oncogene (32) and the cellular *sis* proto-oncogene (33). Moreover, studies linking PDGF to oncogene products, coupled with the findings that human osteosarcomas, fibrosarcomas and glioblastomas in culture synthesize and release biologically active PDGF-like polypeptides into their media, raise the possibility that an inhibitor of this growth factor could represent a useful mechanism for an antineoplastic agent (34–38). SRI 62-834, when added at 10 μM and 1 μM to a human fibroblast cell culture that was then treated with PDGF, caused 100% inhibition of the mitogenic effect of PDGF at the high concentration and 35% inhibition at the lower. At either concentration level, no alteration in cell morphology or cell death was found. These findings suggest that the antimitogenic effect of SRI 62-834 cannot be attributed to the inhibition of thymidine uptake, since it was not inhibited in the absence of PDGF. It is also interesting to note that these concentrations are in the same range as those that gave a good cytotoxic response to the tumors given in Table 1.

To determine whether the PDGF mitogenic inhibition could be receptor mediated, SRI 62-834 was evaluated at 10 μM in the ^{125}I-PDGF receptor assay and found to exhibit only 6% inhibition. At 50 μM, a 100% inhibition of the receptor to ^{125}I-PDGF was achieved. These findings do not support a role for SRI 62-834 as a specific receptor antagonist at the cytotoxic concentration reported in Table 1. It is possible that when administered at higher concentration, SRI 62-834 could perturb the PDGF receptor, alter its interaction with PDGF and exert an antioncogenic effect.

We also investigated the effect of SRI 62-834 on protein kinase C activity, since the binding of PDGF to its receptor has been shown to result in the activation of this enzyme (39). No effect was seen until SRI 62-834 was administered at ca. 1.0 mM (Table 4). This concentration is ca. 100-fold higher than the cytotoxic dose and rules out any involvement of protein kinase C in the antitumor effects of SRI 62-834.

Since SRI 62-834 has a structural resemblance to PAF (Fig. 1), it was evaluated in several tests where PAF has been demonstrated to cause adverse pharmacological results (40). SRI 62-834 at concentrations up to 100 μM did not cause any aggregation of human platelets and demonstrated a protective effect (IC_{50} 19.7 μM) against PAF-induced aggregation. The protective effect of SRI 62-834 against PAF induce aggregation is probably mediated through a receptor mechanism since it inhibited (IC_{50} 27.3 μM) [^{3}H]PAF binding to the human platelet receptor. Administration of SRI 62-834 to guinea pigs at doses up to 2.7 mg/kg ia showed no influence on hematocrit and bronchial resistance. Under similar conditions, 0.001 mg/kg of PAF-18 given IV resulted in marked hematocrit and bronchial changes that led to death. When 2.7 mg/kg of SRI 62-834 was given ia 5 min prior to administration of 75 ng/kg of PAF-18, it failed to protect against the resultant hematocrit and bronchial changes.

In conclusion, we have demonstrated that SRI 62-834 has in vitro and in vivo antitumor activity of the same magnitude as the clinically effective ET-18-OCH_3. The antitumor properties of SRI 62-834 appear to be due to direct and macrophage-induced cytotoxicity. The effect of this agent and related ether phospholipids on PDGF and its relationship to antitumor properties merits additional study.

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Liposomes in Chemo- and Immunotherapy of Cancer[1]

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In this paper, we report on the in vivo behavior of liposomes as a function of their size and composition. It is emphasized that by varying these parameters we can influence not only the rate of blood elimination but also the intrahepatic destination of the liposomes. Thus, we show that small liposomes with diameters well below 100 nm can reach and be internalized by the parenchymal cells of the liver, i.e. the hepatocytes. The rate and the extent at which this occurs depends on the liposomal composition. With respect to the application of liposomes as a drug carrier system in anticancer therapy, we put emphasis on the liver macrophage, i.e. the Kupffer cell, as a target cell. Large liposomes with diameters well over 100 nm exclusively are taken up by these cells as far as hepatic uptake is concerned. By encapsulation within liposomes, a drug may be delivered specifically to these macrophages; this will prevent its rapid excretion from the body and/or undesired accumulation in other cell types. Two examples of the way in which this condition may be exploited are presented. First, we demonstrate the formation of intracellular depots in the macrophages of the cytostatic drug 5-fluorodeoxyuridine (FUdR), thus preventing the rapid metabolism of the drug by the hepatocytes and allowing its sustained release from the macrophages and subsequent uptake by adjacent metastatic tumor cells. Second, we show that the liposome-encapsulated immunomodulator muramyl dipeptide is capable of activating liver macrophages both in vitro and in vivo to a tumor-specific cytotoxic state, and this can result in substantial reduction of metastatic growth in the livers of mice inoculated in the spleen with colon adenocarcinoma cells.
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Liposomes have been considered as a potential drug carrier system for many years (1-3). For this reason, numerous investigators have engaged in attempts to investigate and characterize the in vivo behavior of liposomes (4-6), their modes of interaction with a variety of cell types (7-10) and their effectiveness in the combat of infectious (11-14) or neoplastic (15-18) disease when carrying antibiotic, cytostatic or immunomodulating drugs. The expected modes of action of such therapeutically designed liposomes include direct uptake of the drug-containing liposomes by the target cells, e.g. tumor cells; alteration of the pharmacokinetic properties of the encapsulated drug, such as rate of clearance and tissue distribution, resulting in an improvement of therapeutic index; or uptake by phagocytic cells such as monocytes and macrophages, leading to activation of such cells to a specific tumoricidal state (18-20).

Uptake of liposomes by nonphagocytic cells, including most tumor cells generally is questionable or, at best, of little significance (21,22). Therefore, beneficial effects of liposome encapsulation of a cytostatic drug on its therapeutic efficacy is most likely to result from a favorable effect on its pharmacokinetic characteristics. We show in this paper the dependence of in vivo behavior of liposomes, including intrahepatic distribution, on their size and composition; how this can be applied to modify drastically the pharmacokinetic parameters of a cytostatic drug, 5-fluorodeoxyuridine (FUdR); and that specific uptake of adequately designed liposomes containing muramyl dipeptide by liver macrophages can render these cells in vitro and in vivo cytotoxic to tumor cells and cause them to effectively prevent or reduce metastatic growth in the liver of mice inoculated with colon adenocarcinoma cells.

MATERIALS AND METHODS

Preparation of liposomes. Small unilamellar liposomes (SUV), of the indicated lipid compositions, 25-40 nm in diameter and labeled with encapsulated [^{3}H]inulin in the aqueous space or with a tracer amount of cholesterol [^{14}C]oleate in the lipid phase were prepared by ultrasonication of the appropriate lipid dispersions in buffer as described before (23). Multilamellar liposomes (MLV) with a diameter of approximately 400 nm were prepared from the indicated lipid composition as follows: the lipids, purchased from Sigma Chemical Co. (St. Louis, MO) and stored in chloroform/methanol (8:2) under nitrogen at −20 C, were mixed in appropriate ratios, dried under reduced nitrogen pressure, dissolved in benzene and lyophilized. The lipids then were hydrated in 135 mM NaCl, 10 mM HEPES, pH 7.4 (Hepes/Nace [HN]-buffer) or in HN-buffer containing muramyldipeptide (MDP) or [^{3}H]FUdR and vortexed for 10 min at room temperature. The vesicles formed were sized by extrusion through a series of polycarbonate membranes (Nucleopore) (24) of 1 μm, 0.8 μm, 0.6μm and 0.4 μm pore diameter, respectively. Vesicles were freed from non-encapsulated material on a Sephadex G100 column (Pharmacia [Uppsala, Sweden]). The liposomes were prepared to contain in their aqueous space either [6-^{3}H]-5-fluoro-2′-deoxyuridine (New England Nuclear [Boston, MA], spec. act. 14.7 Ci/mmol) appropriately diluted with unlabeled 5-fluoro-2′-deoxyuridine (Hoffmann-La Roche [Nutley, NJ]) or muramyl dipeptide, from Ciba-Geigy (Basel, Switzerland). Liposome preparations were stored under nitrogen at 4 C and used within two days after preparation.

Isolation of rat liver macrophages. Liver macrophages were isolated by pronase digestion of the liver and purified by centrifugal elutriation as described by Knook and Sleyster (25) and modified by Dijkstra et al. (26). Briefly, the liver was preperfused with Gey's balanced salt solution (GBSS) to remove blood and perfused for three min with 0.2% pronase (Boehringer-Mannheim [Federal Republic of Germany]). The organ was removed, cut into small pieces and incubated in 0.2% pronase for 50 min in the presence of DNase. The cell suspension was washed

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Abbreviations: dTHd, deoxythymidine; EDTA, ethylenediaminetetraacetic acid; FUdR, 5-fluorodeoxyuridine; GBSS, Gey's balanced salt solution; MDP, muramyldipeptide; MLV, multilamellar liposomes; PBS, phosphate-buffered saline; SUV, small unilamellar liposomes.

once, and non-parenchymal cells were separated from non-viable parenchymal cells and remaining erythrocytes by centrifugation on a metrizamide gradient (analytical grade, Nyegaard, Oslo, Norway) (0.7%) for 15 min, 1500 × g. The top layer containing the nonparenchymal cells was washed once and resuspended in 5 ml GBSS containing DNase. These cells were fractionated into a macrophage and an endothelial cell fraction by elutriation centrifugation (Beckman Instruments [Palo Alto, CA], elutriation rotor type JE-6). The cell suspension was flushed into the rotor at a flow rate of 20.5-21.0 ml/min, rotor speed 2500 rpm, at 4 C. At this flow rate, lymphocytes and endothelial cells were flushed out in 250 ml GBSS. The macrophages were collected at a flow rate of 46.5 ml/min in 150 ml GBSS, concentrated by centrifugation at 700 × g for 10 min and resuspended in culture medium containing 20% fetal calf serum; 2.5 × 10^5 liver macrophages in 200 μl culture medium were seeded per well in 96-well microtiter plates (Costar, Cambridge, MA) to obtain a monolayer of liver macrophages.

Intrahepatic distribution of liposomes. Liposomes, either SUV containing [^{3}H]inulin or MLV containing [^{3}H]FUdR, were injected into the penile vein of ether-anesthesized male Wistar rats varying in body weight between 170 and 260 g. Liposome elimination from blood was determined by blood sampling from the tail vein. The injected dose was 0.1 mmol of total lipid per kg body weight. For determination of liposome uptake by total liver, the organ was perfused in situ via the portal vein with isotonic saline at 37 C to remove blood and homogenized in a Potter-Elvehjem tube; aliquots of the homogenate were assayed for radioactivity. Intrahepatic distribution of liposomes was determined by measuring radioactivity content of the isolated parenchymal and nonparenchymal liver cell fractions, assuming 450 × 10^7 hepatocytes and 194 × 10^7 nonparenchymal cells per kg body weight (27). Parenchymal cells (hepatocytes) were isolated after perfusion of the liver with collagenase and nonparenchymal cells (Kupffer cells and endothelial cells) after digestion of the liver with pronase as described above.

Uptake of liposomes by Kupffer cells in vitro. Kupffer cells were isolated and purified as described above and allowed to attach to 35 mm plastic petri dishes at a density of 1.5 × 10^6-2.0 × 10^6 cells per dish under the conditions described previously (26). After at least 24 hr, the cells were incubated as reported (26) with SUV (70 nmol of liposomal lipid) of the compositions given in Table 1.

In vitro cytolytic assay. Macrophage-mediated cytolysis was assessed by a radioactivity release assay. One day after isolation, isolated liver macrophages in monolayer culture were incubated with free or liposome-encapsulated immunomodulators. Target tumor cells in the exponential growth phase were radiolabeled by a 20-hr incubation in a medium containing 0.3 μCi [^{3}H]deoxythymidine (dThd) per ml. The cells then were washed free from radioisotope and cold-pulsed by incubation in fresh medium for three-four hr to deplete cytoplasmic pools of [^{3}H] dThd and minimize spontaneous release of label. Subsequently, the cells were washed twice with phosphate-buffered saline pH 7.4 (PBS) at 37 C, to remove unbound radiolabel, harvested by short trypsinization (0.05% Difco trypsin, 0.2% ethylenediaminetetraacetic acid (EDTA), for 45 sec at 37 C), washed four times with PBS and resuspended in culture medium at a concentration of 10^5 cells/ml. Four hr after the addition to the macrophage monolayers of 100 μl culture medium (10% fetal calf serum) containing the immunomodulator(s), 100 μl medium containing 10^4 [^{3}H]dThd-labeled tumor cells were added per well. At this ratio of macrophage to target cell (25:1), untreated macrophages were not cytotoxic to neoplastic cells. Radiolabeled target cells also were plated alone as an additional control. Forty-eight hr after addition of the tumor cells, the supernatants were collected, and the radioactivity was measured in a liquid-scintillation counter. Specific cytolysis was calculated as follows:

$$\% \text{ specific cytolysis} = 100 \times \frac{a - b}{c - b}$$

in which a, dpm in supernatant of tumor cells cocultured with test macrophages; b, dpm in supernatant of tumor cells cocultured with control macrophages; c, dpm in total amount of tumor cells added per well.

In vitro cytostatic assay. Macrophage-mediated inhibition of the proliferative capacity of tumor cells was assessed by measuring [^{3}H]-dThd incorporation into the DNA of cocultured tumor cells. After a four-hr

TABLE 1

Uptake of Small Liposomes by Liver and Liver Cells In Vivo and Liver Macrophages In Vitro[a]

Liposome composition[b]	% of injected dose in				nmol/10^6 Kupffer cells	
	Blood	Whole liver	P-cells	NP-cells	In vivo	In vitro
CH:PC (1:1)	14.0 ± 5.5	45.4 ± 7.0	54.1 ± 5.6	2.3 ± 1.1	4.8	3.0
CH:SM (1:1)	62.9 ± 2.3	6.3 ± 2.3	5.4 ± 1.9	1.2 ± 0.7	2.4	1.6
CH:PC:PS (5:4:1)	3.0 ± 1.7	54.5 ± 3.0	57.3 ± 5.7	8.4 ± 3.0	17.2	29.4
CH:SM:PS (5:4:1)	29.6 ± 7.5	28.4 ± 8.6	20.7 ± 5.1	7.8 ± 2.0	16.0	15.3

[a]Small unilamellar liposomes, labeled with encapsulated [^{3}H]inulin and composed as indicated, were injected intravenously (0.1 mmol of lipid per kg body weight) into male Wistar rats. After 5.5 hr, the liver was perfused, and parenchymal (P) or nonparenchymal (NP) cells were isolated by collagenase or pronase digestion, respectively. Radioactivity in blood, whole liver and cell fractions was determined. Results are given as % of injected dose (± SD) or as nmol of total liposomal lipid per 10^6 (Kupffer) cells. In the last column, results are presented of in vitro experiments with isolated Kupffer cells in maintenance culture; 1.9 × 10^6 cells were incubated for three hr with 70 nmol total lipid of small unilamellar vesicles of the indicated composition and labeled with a trace amount of cholesteryl-[^{14}C]oleate.
[b]CH, cholesterol; PC, egg phosphatidylcholine; SM, bovine brain sphingomyelin; PS, bovine brain phosphatidylserine.

incubation of the macrophage monolayers with 100 μl culture medium containing immunomodulators, 100 μl medium containing 10^4 tumor cells were added per well. Tumor cells alone were plated as an additional control. After 24 hr cocultivation, 0.01 μCi [^{3}H]-dThd was added per well. Twenty-four hr after the addition of label, the cultures were washed three times with PBS (4 C), and the adherent viable cells were lysed with 0.2 ml of 0.5 M NaOH. The radioactivity of the lysate was measured in a liquid-scintillation counter, and the inhibition of tumor cell proliferation was calculated as follows:

$$\% \text{ inhibition} = 100 \times (1 - \frac{x}{y})$$

in which x, dpm in tumor cells cocultured with test macrophages; y, dpm in tumor cells cocultured with control macrophages.

RESULTS AND DISCUSSION

The rate of elimination from the blood of IV-administered liposomes depends strongly on size and composition of the liposomes. Large liposomes with diameters larger than ca. 0.1–0.2 μm are eliminated rapidly, i.e. with half-lives in the order of 5–15 min. Within this range, rates may depend on lipid composition, such as presence of charged lipids and cholesterol content (28). Small liposomes with diameters below 0.1 μm are cleared much more slowly, with half-lives ranging from one hr up to as much as 15 or 20 hr (29,30). Also, in this case lipid composition largely determines the precise value of the elimination rate, e.g. negatively charged vesicles are eliminated faster than similarly composed vesicles carrying no net charge. Remarkably, elimination rates also may be affected by more subtle differences in lipid composition. For example, small unilamellar vesicles composed of equimolar amounts of cholesterol and sphingomyelin are cleared extremely slowly with a half-life of ca. 12–15 hr whereas vesicles in which the sphingomyelin has been replaced by egg phosphatidylcholine, thus exposing an identical polar head group at their surface, are cleared almost five-fold more rapidly (23). Since resident macrophages in liver and spleen mainly have been held responsible for blood elimination of liposomes, it would seem straightforward that the rate of liposome elimination is related directly to the rate of uptake by these macrophages. Indeed, we observed a correlation between these two parameters when measuring the rate of uptake of differently composed small unilamellar liposomes by rat liver macrophages (Kupffer cells) in vitro (ref. 23, Table 1).

Although resident macrophages, including those in the liver, do display a strong affinity for most types of liposomes, other cells also have been shown to be able to participate in the in vivo clearance of liposomes from the blood. In particular, we found the parenchymal cells of the liver, i.e. the hepatocytes, very active in the uptake of small liposomes. Especially neutral liposomes and of those specifically, the egg-phosphatidylcholine/cholesterol vesicles, showed a relatively high affinity towards the hepatocytes, culminating in relative extents of uptake by these cells of as much as 95% of the total liver uptake as opposed to only a 5% uptake by the Kupffer cells (23).

The discrimination between large and small liposomes with respect to their intrahepatic disposition is attributed to the condition in which the liver's endothelial lining of the sinusoidal spaces that separates the parenchymal cells from the blood compartment is perforated. The liver endothelial cells possess numerous open fenestrations with an average diameter of about 100 nm, allowing passage of >100 nm liposomes and preventing access of larger liposomes to the parenchymal cells (31). In this paper, which is devoted to the use of liposomes in anticancer therapy, we will focus on those liposome species that are taken up by the liver macrophages. Internalization of liposomes by these cells can be exploited in the combat of metastatic growth in the liver by a duplicate approach. First, by encapsulation in liposomes cytostatic drugs may be accumulated at the tumor growth site, i.e. the liver, and form an intracellular depot in the Kupffer cells from which the drug becomes available in a time-dependent way at a rate that can be controlled by adequately manipulating the liposomal composition. The latter will

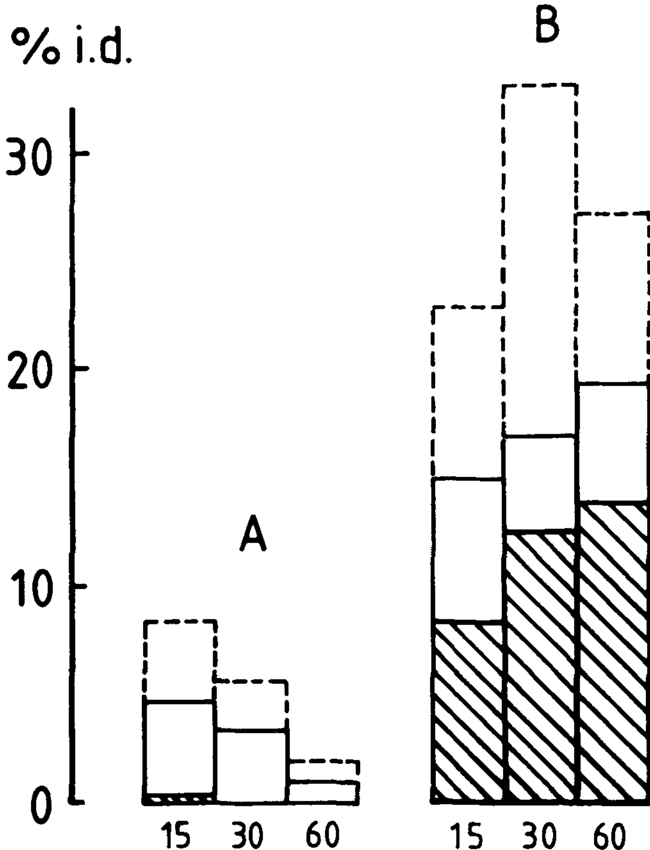

FIG. 1. Multilamellar liposomes containing [6-^{3}H]5-fluoro-2′-deoxyuridine (FUdR) were prepared from sphingomyelin, cholesterol and phosphatidylserine in a 4:5:1 molar ratio as described in Methods. These liposomes were injected at a dose of 50 μmol of total lipid, encapsulating 13 mg of FUdR, per kg body weight. Alternatively, rats were injected with the same amount of unencapsulated [^{3}H]-FUdR. At the times indicated, livers were perfused, and parenchymal and nonparenchymal cells were isolated by collagenase perfusion. Uptake in total liver and the two liver cell fractions was determined and is presented as percent of injected dose. The total lengths of the bars, including the stippled parts, represent uptake by whole liver; the open solid parts, the hepatocyte uptake; the hatched parts, the nonparaenchymal cell uptake. Results are means of three experiments agreeing within 10%.

be a major factor in determining the susceptibility of the liposomal lipid constituents towards lysosomal phospholipases, hence the rate of release of an encapsulated solute (32). Figure 1 shows an example of the alteration of in vivo behavior of a cytostatic drug as a result of its encapsulation in liposomes. The drug in this case was FUdR, a derivative of the widely used 5-fluorouracil. The latter drug particularly has been applied for several years in the therapeutic treatment of patients suffering from liver metastases originating from colorectal cancers, a major cause of cancerous death in the Western world. Both drugs are only moderately effective; one of the reasons is that they rapidly are taken up and metabolized by the parenchymal cells of the liver. Encapsulation of the drugs within liposomes should prevent this because the drug is expected to be directed to the Kupffer cells when administered in encapsulated form. As can be seen in Figure 1, this is previsely what happens. The radiolabeled drug, i.e. the deoxyuridine derivative since the fluorouracil is not sufficiently polar to be retained in liposomes, when administered in free form (A) is found to accumulate only to very limited extents in the liver. Fifteen min after injection, less than 10% of the injected dose is recovered in this organ (stippled bars); when parenchymal and nonparenchymal cells are isolated from these livers, only about half of the total liver-associated drug is recovered, virtually all of which is in the parenchymal cells (open full bars); only negligible amounts of drug can be found in the nonparenchymal cell fraction. Sixty min after injection, nearly all drug radioactivity has disappeared from the liver. However, when the FUdR is injected encapsulated in liposomes an entirely different situation is achieved (B). First, the extent of accumulation is substantially higher; 30 min after injection, more than 30% of the injected dose is recovered in the whole liver. Second, the drug is retained much longer in the liver; at 60 min, still more than 25% of the injected dose is present as opposed to less than 2% for the free drug at that time. Finally and probably most importantly, the intrahepatic distribution of the drug is quite different from that of the free drug. While the latter was found virtually only in the hepatocyte fraction of the encapsulated drug, about two-thirds of the total liver-associated fraction (hatched bars) is found in the nonparenchymal cells, i.e. the Kupffer cells (endothelial cells were repeatedly shown not to participate in liposome uptake whatsoever). The 25-40% that is found in the hepatocyte fraction has to be ascribed partially to contamination of this fraction with nonparenchymal cells and partly to the presence of a substantial amount of relatively small vesicles in the vesicle preparations (extruded MLV) used to encapsulate the drug. We recently observed that liposome preparations (MLV and LUV) subjected to forced filtration through polycarbonate filters may, depending on the lipid composition, contain substantial amounts of vesicles that are much smaller than the pore diameter of the filters. The consistent discrepancies between total liver uptake and the sum of the uptake by the separate cell fractions both for the free and the encapsulated drug have to be attributed to the release of drug that takes place during the time required to isolate the cells.

Our experiments demonstrate that by encapsulation of FUdR in liposomes, the pharmacokinetic characteristics of the drug substantially are altered. The rate of elimination from the blood is somewhat decreased; liver uptake and retention drastically are increased; and the intrahepatic distribution is shifted from the hepatocytes to the Kupffer cells. Obviously, the question now is does this improve the therapeutic efficacy of the drug? This remains to be seen. If so, it likely will depend on the rate at which the drug becomes available from the cell. In turn, this will depend on the rate of intracellular degradation and thus on the lipid composition of the liposome. For example, it is to be expected that encapsulation of the drug in liposomes of phospholipase A-resistant dialkyl phospholipids rather than diacylphospholipids will lead to enhanced retention times in the liver and concomitantly to slower rates of release of the drug.

It is with those considerations that we are studying the intracellular metabolic fate of liposomes as a function of liposome composition and with special emphasis on the Kupffer cells as the cell type most extensively involved with in vivo clearance of liposomes from the blood. In addition, considering the intralysosomal localization of the liposome-encapsulated drug, it will be important to establish how rapidly and in what chemical form the released drug will leak out of the Kupffer cell lysosomes. The presence or absence of enzymes in these cells capable of metabolizing the drug yet has to be established.

A basically different approach that may be taken to eradicate metastatic growth in the liver by means of liposomes is by way of activation of the Kupffer cells to tumorcytotoxicity, i.e. by exploiting part of the body's natural defense mechanism against the proliferation of (metastatic) tumor cells. Fidler and his associates have shown that certain biological response modifiers when encapsulated in liposomes have a much higher macrophage-activating potency than the free substance and thus can cause impressive reduction or even complete eradication of established lung metastases (18,32).

Encouraged by the results of Fidler and coworkers, we embarked on an attempt to follow a similar approach for liver metastasis. From earlier work, we knew that isolated Kupffer cells in monolayer culture are able to endocytose and degrade substantial amounts of liposomes (26,33,34). Uptake rates in those experiments amounted to ca. 2 nmol of lipid per hour per 10^6 cells, a value that we also found in the present work using liposomes of slightly different composition. First, we established the ability of rat liver macrophages (Kupffer cells) to become activated to a tumoricidal state as a result of an exposure to liposomes containing the immunomodulator MDP. Kupffer cells were isolated, purified and allowed to attach to microtiter plates. One day after attachment to the plates, the cells were incubated with various amounts of liposomes containing encapsulated MDP. The extent of cytotoxicity attained by the cells (i.e. cytolysis) was assessed by measuring release of [^{3}H]-dThd from tumor cells (B16 melanoma or C26 colon adenocarcinoma) that were labeled in their DNA with [^{3}H]-dThd (35). Alternatively, we determined cytostasis by measuring the effect of the activated liver macrophages on the proliferative capacity of the tumor cells, assayed as [^{3}H]-dThd incorporation. Table 2 shows that the Kupffer cells, upon incubation with MDP-containing liposomes, acquire strong cytolytic and cytostatic properties in a dose-dependent way. With the highest amount of liposomes (ca. 0.8 μmol of liposomal lipid per 10^6 cells), the extent of cytolysis amounted to

TABLE 2

Cytolytic and Cytostatic Activity of Liver Macrophages Following In Vitro Incubation With MDP-containing Liposomes[a]

	Cytolysis			Cytostasis		
Liposomal lipid added (nmol)	200	100	50	200	100	50
Macrophages treated with MDP liposomes	1306 ± 3 (56)	1082 ± 84 (41)	929 ± 64 (31)	121 ± 16 (99)	220 ± 79 (98)	962 ± 21 (89)
Macrophages treated with control liposomes	485 ± 42 (1)	455 ± 28 (0)	439 ± 111 (0)	9367 ± 56 (1)	9734 ± 508 (0)	9092 ± 868 (0)
Macrophages treated with medium only		463 ± 43			9105 ± 572	
No macrophages; B16 cells only		420 ± 21			8668 ± 556	

[a]Rat liver macrophages (Kupffer cells) were isolated and allowed to attach to culture plates; one day after isolation 2.5 × 10^5 cells were incubated for four hr with multilamellar liposomes composed of cholesterol, egg phosphatidylcholine and dicetylphosphate (5:4:1, molar ratio) and, when applicable, containing 1 μg MDP/μmol of lipid. 10^4[^{3}H]deoxythymidine-labeled B16 melanoma cells were added; after 24 hr, release of ^{3}H radioactivity into the medium was measured. Alternatively, unlabeled tumor cells were added to the activated macrophages and after 24 hr of coculturing [^{3}H]deoxythymidine (^{3}HdThd) incorporation into tumor cells was measured. Data represent radioactivity (dpm) released from (cytolysis) or incorporated into (cytostasis) tumor cells. All values are means of triplicate experiments ± SD. Numbers in parentheses are % specific cytolysis or, in case of cytostasis, % of inhibition, both calculated as described in Methods.

well over 50% (corrected for proper control values) while cytostasis was nearly complete under those conditions. When the macrophages were incubated with the same amounts of MDP in a free, unencapsulated form, no significant cytolysis or cytostasis could be detected. Interestingly, we observed that a fixed amount of MDP when encapsulated in a certain quantity of lipid resulted in much higher extents of cytotoxicity than when it was encapsulated in a 10-fold smaller quantity of lipid (35). This may suggest that the rate of intracellular degradation of the liposomes is correlated with the potency of the liposome-encapsulated MDP and thus may provide a clue as to the mechanism of action of this immunomodulator. In attempts to activate the liver macrophages in vivo as a result of uptake of MDP-containing liposomes following IV administration, we generally observed that when the Kupffer cells were isolated 18 hr after liposome administration extents of tumorcytotoxicity were significantly lower than those obtained in vitro, particularly when the B16 melanoma cells were used as target cells. Remarkably, we found no clear dose-response effect; MDP encapsulated in a small amount of liposomal lipid produced the same degree of cytotoxcity as the same amount of MDP in a 10-fold higher quantity of liposomes. Similarly, no significant differences in tumorcytotoxicity were observed between various subpopulations of Kupffer cells, which were isolated according to size, despite substantial differences in liposome uptake between those subpopulations (36). Also, this observation suggests a lack of dose-response relationship. An alternative, although rather improbable explanation would be that the low liposome uptake by the smaller cell sizes is compensated precisely by a high potential of these cells to become tumoricidal. A satisfactory explanation for these observations obviously has to await further experimentation. However, it is clear that the potential to become activated to tumorcytotoxicity is a property of the total Kupffer cell population; this should be considered a favorable condition for the in situ activation of the Kupffer cells in order to eradicate or prevent metastatic growth throughout the liver.

In spite of many unanswered questions with respect to the mechanism of action of the liposome-encapsulated MDP under in vivo conditions, we did observe a definitely favorable effect of this therapeutic modality on the growth of C26 adenocarcinoma cells in the liver following intrasplenic inoculation of these tumor cells and IV administration of MDP-containing liposomes. While in 95% of the control animals the livers were studded with numerous metastatic foci, the livers of 40% of the treated animals were completely free of metastases; another 40% contained fewer than four metastatic nodules.

In conclusion, this paper serves to show that liposomes have the potential to be used as a drug carrier system to both the parenchymal and the nonparenchymal cells of the liver. Of the nonparenchymal cells, it is only the Kupffer cell fraction, i.e. the macrophages, that is capable of internalizing the liposomes; we show how this can be exploited to prevent the development of metastatic growth in the liver. By encapsulation within liposomes, the pharmacokinetic properties of a cytostatic drug such as FUdR can be altered so as to create a relatively long-lasting intracellular depot from which the active drug is released in the immediate vicinity of the target cells. On the other hand, the liposomes may be applied to carry an immunomodulator such as MDP, which in free form rapidly would be cleared through the kidneys, very effectively to the liver macrophages, thus causing these to become activated to a tumoricidal state in which they are able to kill low tumor burdens. Not only did we show that liver macrophages are susceptible to this kind of activation both in vitro and in situ, we also demonstrated the therapeutic effectiveness of this approach in a liver-metastasis model in mice.

ACKNOWLEDGMENTS

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Cytostatic Activity of Synthetic O-Alkylglycerolipids[1]

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A series of halogen-containing alkylglycerolipid analogs has been checked for their cytostatic activity both in vitro and in vivo. The compounds included alkyldeoxyhaloglycerols (I), alkyldeoxyhaloglycerophosphocholines (II), and alkyldeoxyhaloglycerophosphoric acids and alkyl esters (III). While compounds I and III were moderately active, compounds II were found to have a strong inhibitory effect on the proliferation of Ehrlich ascites tumor cells in vitro. Cell growth inhibition of 50% or more was found mainly in the late S- or G_2-phase of the cell cycle as revealed by flow cytometry. Alkyl lysophospholipid analogs II and cholesterol form liposomes with high encapsulation efficiency and low permeability for entrapped substances. Compounds II were active against Lewis lung carcinoma in mice when applied in free form or as liposomes.
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The structural modification of metabolites and other naturally occurring compounds often has yielded substances with interesting biological effects. Thus, by the introduction of a halogen atom, e.g. fluorine or chlorine, into nucleosides, antimetabolites of nucleic acid metabolism, endowed with cancerostatic properties, have been obtained (1). Since recent evidence indicates that phospholipid metabolism is involved in the regulation of cell proliferation (2,3), it has been of considerable interest to modify the structure of phospholipids and their natural precursors to obtain antimetabolites of phospholipid metabolism as potential cancerostatically active substances. We have prepared a series of halogen-containing neutral lipid and phospholipid analogs and checked their cytostatic activity.

Table 1 shows the types of compounds investigated, including analogs of *O*-alkyl(acyl)- and di-*O*-alkyl(acyl)-glycerol, *O*-alkylglycerophosphoric acid, *O*-alkylglycero-

TABLE 1

Chemical Structure of Halogen-Containing Neutral Lipid and Phospholipid Analogs

Structures: CH_2-O-X / CH-Y / CH_2-O-Z; CH_2-Y / CH-O-X / CH_2-O-Z; CH_2-O-X / CH-O-Z / CH_2-Y

Analogs of	X	Y	Z
O-Alkylglycerols	$C_{16}H_{33}$	Cl,F	H
Di-*O*-alkylglycerols	$C_{16}H_{33}$	CF_3CH_2O	H
O-Acylglycerols	Acyl	Cl,F	H
Di-*O*-acylglycerols	Acyl (C_{12-22})	Cl,F	Acyl (C_{12-22})
O-Alkylglycerophosphoric acids	$C_{16}H_{33}$	Cl,F	P(=O)(OH)OH
O-Alkylglycerophosphoric alkyl esters	$C_{16}H_{33}$	Cl,F CF_3CH_2O	P(=O)(O(CH_2)$_n$H (Br,Cl))OH
O-Alkylglycerophosphocholines	$C_{16}H_{33}$	Cl,F CF_3CH_2O	P(=O)(O(CH_2)$_n\overset{+}{N}Me_3$ (NMe_2))O^-
			n = 2,3

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Abbreviations: ALP, alkyl lysophospholipids; CF, 6-carboxyfluorescein; CH, cholesterol; DG, diacylglycerol; EAT cells, Ehrlich ascites tumor cells; EPC, egg phosphatidylcholine; HEPC, hydrogenated egg phosphatidylcholine; ip, intraperitoneal(ly); IP_3, inositol-1,4,5-triphosphate; MLV, multilamellar vesicles; PA, phosphatidic acid; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-diphosphate; PKC, proteinkinase C; SUV, small unilamellar vesicles.

TABLE 2

Growth Inhibitory Effects of Selected *O*-Alkylglycerophospholipid Analogs on Ehrlich Ascites Tumor Cells In Vitro[a]

Compounds	I 50%[b] (μM)	% inhibition at 100 μM
A. *O*-Alkylglycerophosphoric acid analogs		
X, Y, O-P(=O)(OH)OH; X: OR, OR, F, Cl; Y: F, Cl, OR, OR	>100	>50
B. *O*-Alkylglycerophosphoric alkyl ester analogs		
1(a): O-R, F, O-P(=O)(OH)-OCH_2CH_2Cl (H)	40	96
2(a): O-R, F, O-P(=O)(OH)-OCH_2CH_2Br (H)	56	75
3: O-R, Cl, O-P(=O)(OH)-OCH_2CH_2Br; 4: F, O-P(=O)(OH)-OCH_2CH_2Br, O-R	>100	42
5: O-R, O-CH_2CF_3, O-P(=O)(OH)-OCH_2CH_2Br	>100	47
6: O-CH_2CF_3, O-R, O-P(=O)(OH)-OCH_2CH_2Br	66	63
7: O-R, CH_2, O-P(=O)(OH)-OCH_2CH_3	>100	15
8: R-O-P(=O)(OH)-OCH_2CH_2Br	>100	3

(continued on next page)

TABLE 2 *(continued)*

	I 50% (μM)	% inhibition at 40 μM
C. *O*-Alkylglycerophosphocholine analogs		
1: O-R / Cl / O-P(=O)(Chol)O^-	22	73
2: Cl / O-P(=O)(Chol)O^- / O-R	24	75
3: Cl / O-R / O-P(=O)(Chol)O^-	20	76
4: O-R / O-CH_2CF_3 / O-P(=O)(Chol)O^-	9.5	100
5: O-R / O-P(=O)(Chol)O^- / O-CH_2CF_3	22	78
6: O-CH_2CF_3 / O-R / O-P(=O)(Chol)O^-	24	68
7: O-$C_{18}H_{37}$ / O-CH_3 / O-P(=O)(Chol)O^- (ET-18-OCH_3, ref. 4)	17	82
R = $C_{16}H_{33}$, Chol = $OCH_2CH_2\text{-}\overset{+}{N}(CH_3)_3$		

[a]All samples were incubated in duplicate. Differences were below 5% of the mean value.
[b]Concentration for 50% inhibition of growth rate.

phosphoric alkyl ester and *O*-alkylglycerophosphocholine (racemic forms). These analogs are characterized structurally by the presence of the long-chain alkyl (or acyl) group and a halogen (partially instead of an OH-group) at various positions of the molecule.

The latter compounds are of special interest because of their structural similarity to platelet activating factor (PAF) as well as to other alkyl lysophospholipids (ALP) with antitumor activity (4,5). They represent lysophosphatidylcholine analogs,which do not serve as substrates for lysophospholipase and acyltransferases.

MATERIALS AND METHODS

Syntheses, isolation, purification and analytical data of the halogen-containing lipid analogs investigated have been described (6–11). Also, the cytostatic effects of alkyl(acyl)glycerol analogs have been determined (12). For estimation of the in vitro activity of phospholipid analogs, EAT cells were obtained from the intraperitoneal (ip) cavity of mice five to seven days after transplantation.

Erlich ascites tumor (EAT) cells (5×10^4/0.5 ml) were cultured in suspension (Eagle-MEM) in the presence and absence of the substance to be tested. After 48 hr, the

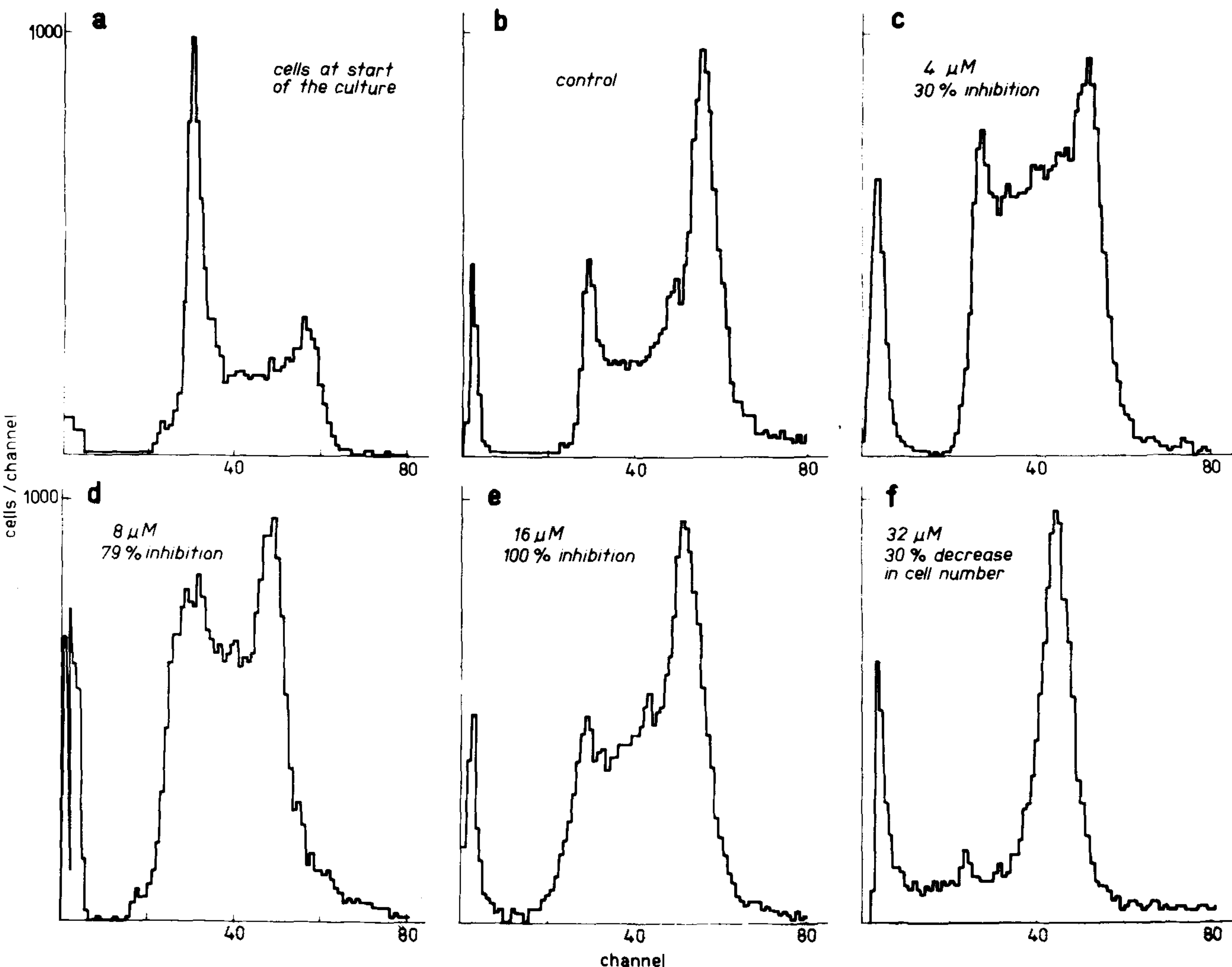

FIG. 1. Flow cytometry study on the influence of 2-chloro-2-deoxy-1-*O*-hexadecyl-glycero-3-phosphocholine on EAC cells in vitro (24 hr suspension culture). The increase in cell number in the controls was 91%.

increase in cell number, which was about five-fold for the untreated controls, was determined. For flow cytometry studies, EAT cells were obtained from mice eight days after transplantation and cultured in suspension for 24 hr. The cells were fixed in ethanol and stained with a mixture of ethidium bromide and olivomycin. The DNA-distribution histograms were obtained with a Phywe ICP-11 instrument. The PIP and PIP_2 turnover in human erythrocyte membranes was investigated according to Müller et al. (13). The membranes were incubated with γ-^{32}P-ATP in the presence and absence of the lipid analogs for 15–20 min, followed by acidic lipid extraction, thin layer chromatography (TLC) and scintillation counting.

Proteinkinase C (PKC) was isolated from porcine brain homogenate as described by Uchida and Filburn (14). The influence of halo analogs of ALP on PKC activity was determined using histone H1 as substrate. Multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) were produced as follows: the solutions of phospholipids (40 μM) and cholesterol, in a 1:1 molar ratio (chloroform), were mixed in a round-bottom flask and evaporated. To the film obtained, 1.5 ml of phosphate-buffered saline (PBS), pH 7.2, or 1.5 ml 0.2 M CF solution were added. The suspension was shaken at room temperature for 12 hr to give MLV. SUV were obtained by subsequent sonication under nitrogen for 16 min followed by centrifugation at 100,000 × g for 1 hr to remove MLV and titanium particles.

In the presence of CF, the supernatant fluid was subjected to Sephadex-G 50 column chromatography (10 ml gel) to remove extravesicular CF. Entrapment efficiency and leakage on storage were measured (15). For the determination of antineoplastic activity in vivo, the murine tumor models, P 388 leukemia and Lewis lung carcinoma, all growing in C57Bl/6 × DBA/2 (BDF_1) mice, were used. The ascitic cells of the leukemia (5 × 10^5/mouse) were inoculated ip or IV; treatment began one day later. The Lewis lung carcinoma was inoculated in the left hind footpad of the animals (brei, 1:4 diluted with Hank's balanced salt solution, 0.05 ml/mouse). In half the animals, the primary tumor was surgically removed on day 10. Drug treatment was accomplished either on days 4–7 without surgery or on days 11–14 after surgery. In this way, both

the effect on the primary tumor and the lung metastases could be followed. For the estimation of toxicity, body weight difference between the first treatment day and after therapy was determined. The substances were administered in a dose of 0.5 or 5 mg/kg/d. With these doses, no signs of toxicity were observed. Statistical significance was determined by the U-test of Mann and Whitney.

RESULTS AND DISCUSSION

Cytostatic activity in vitro. The effects of selected halo analogs of alkyl(acyl)glycerols on the growth of EAT cells in vitro have been described (12). The diacyldeoxyhaloglycerols have been found to be inactive; the monoalkyl(acyl)deoxyhaloglycerols showed a fairly strong activity. The strongest effects were obtained with the glycerol ethers, e.g. the different structural isomers of hexadecylchlorodeoxyglycerol (12).

In Table 2, the cytostatic effects of selected halogen-containing *O*-alkylglycerophospholipid analogs and some related compounds on EAT cells in vitro are shown.

While the compounds of Type A at 100 μM are hardly active on EAT cells in vitro, some of the alkylglycerophosphoric ester analogs of Type B, especially the 1-*O*-alkyl-2-deoxy-2-halo derivatives B1-3 are moderately active. Since the alkyl esters B1a and 2a show a similar activity as the halo-alkyl esters B1 and 2, the effect is not due to the alkylating properties of the 2-halo-ethyl group. The presence of the glycerol moiety seems to be important for the cytostatic effect (loss of activity of B8). The lysophosphatidylcholine analogs (Type C) exhibit strong cytostatic activity against EAT cells, being in the same order as that of compound C7 investigated by Munder et al. (4). The analog 4 is the most effective compound of Type C. Almost the same efficiency is found for the structural isomers C1-3, 5 and 6. The presence of long-chain alkyl as well as phosphocholine, is of major importance for the inhibitory action, while the relative position of these groups and the presence of additional substituents (halogen, short-chain alkyl) is less relevant.

The effectivity is not due to the choline group, since several N,N-dimethylethanolamine derivatives, e.g. analogs of C1 and C2, are also active (not shown). P. G. Munder has performed further experiments with other tumor cells. He has shown that the direct cytotoxic effect on tumor cells (oesophagus carcinoma cells, Meth A sarcoma cells) as well as the enhancement of cytotoxic properties on bone marrow macrophages (Meth A sarcoma cells), by halogen-containing ALP analogs are of the same efficiency as the reference compound ET-18-OCH_3.

Flow cytometry studies. To elucidate the influence of the alkyl lysophospholipid analogs on the passage of the cells through the cell cycle, the effect of 2-chloro-2-deoxy-1-*O*-hexadecylglycero-3-phosphocholine was studied by flow cytometry (Fig. 1).

Cell numbers in the controls approximately doubled in 24 hr. The corresponding histograms (Fig. 1,a and b) show that a majority of cells passed through the greater part of the cycle a second time during this period, entering the G_2-phase. In contrast, cells inhibited in their proliferation by 30%, 80% or completely (Fig. 1, c, d and e) are found in the late S- or G_2-phase of the first cycle. It follows that under treatment, the cells do not remain simply in the phase in which they were at the beginning of the experiment; they pass through the cycle at a slower rate. However, a further doubling of the inhibitor concentration results in some cell loss (Fig. 1, f). Under these conditions, one single peak (late S-phase) is found; this could be explained by cell lysis. Similar distribution patterns were obtained in all of the four experiments.

Mechanism of action. The cytostatic activity of ALP has been explained by a serious disturbance of the membrane phospholipid metabolism of the tumor cell and by the generation of tumoricidal macrophages (4,5). Contrary to normal cells, most tumor cells are thought to lack an *O*-alkyl cleavage system (16,17). This difference in enzyme pattern may cause a predominant accumulation of ALP in tumor cells and a selective antitumor effect (4,5). The higher levels of ALP may disturb membrane function, including lipid-related enzymes (17) and phospholipid-dependent protein kinase C (18). Moreover, ALP were found to induce differentiation of cultured human and murine myeloid leukemia cells (19). The induction of interferon is another biological effect worth mentioning in regard to antitumor activity. Initial experiments have indicated that some halo analogs of ALP and of *O*-alkylglycerophosphoric esters cause an increase in interferon production of human leukocytes stimulated by various mitogens (20).

The halogen-containing ALP analogs 1-3, 5 and 6 (Table 3, C) were found to stimulate human and rabbit platelets. At 50-500 μM, a concentration-dependent platelet aggregation is triggered in human platelet-rich plasma. Distinctly lower concentrations up to 10 μM activate the platelets in rabbit platelet-rich plasma. Moreover, the halo analogs enhance aggregation and release reactions triggered by suboptimal concentrations of ADP (21). It recently has been demonstrated (3) that an increased turnover of phosphatidylinositol (PI) and its phosphorylated derivatives, e.g. PI-4-phosphate (PIP) and PI-4,5-diphosphate (PIP_2), is an early event in the initiation of cell proliferation and other cellular responses.

TABLE 3

Effects of Various Concentrations of *O*-Alkyl(acyl)glycerol Analogs on the Incorporation of ^{32}P-Phosphate into Phosphatidylinositol-4-phosphate (PIP) and Phosphatidyl-inositol-4,5-diphosphate (PIP_2) of Human Erythrocyte Membranes

Concentration (μM)	Incorporation of radioactivity % of control			
	O-$C_{16}H_{33}$ / O-CH_2CF_3 / OH		O-$COC_{15}H_{31}$ / F / OH	
	PIP	PIP_2	PIP	PIP_2
0.75	111	86	85	70
2.25	101	85	99	93
7.50	92	92	91	93
22.50	85	78	87	57
75.00	54	53	56	39

A stimulus-mediated PIP_2-breakdown releases two second messengers, 1,2-di-*O*-acylglycerol (DG) and inositol-1,4,5-triphosphate (IP_3). DG and the elevation of Ca^{++} synergistically activate Ca^{++}- and phospholipid-dependent protein kinase C (PKC), which is further involved in catalyzing phosphorylation of membrane protein. Moreover, DG analogs recently have been found to stimulate a rapid increase in the levels of PIP and PIP_2 (22). Thus, the ability of DG to function as a bioregulator in phosphoinositide turnover and PKC activation, as well as the phospholipid dependence of PKC activity, prompted us to investigate (a) the influence of synthetic mono- and dialkyl(acyl)glycerols on the PI metabolism and (b) the effects of *O*-alkylglycerophosphocholine analogs on the PKC activity.

Table 3 shows the dose-dependent inhibition by 1-*O*-hexadecyl-2-(2,2,2-trifluorethyl)glycerol and 2-deoxy-2-fluoro-1-*O*-palmitoylglycerol on the incorporation of [^{32}P]-phosphate into PIP and PIP_2 of human erythrocyte membranes. This demonstrates for the first time that several monoalkyl(acyl)deoxyhaloglycerols are inhibitors of PIP and PIP_2 turnover in human erythrocyte membrane. Also, a number of other selected halogen-containing alkyl(acyl)-glycerol analogs inhibited this process up to about 40% at 50 μM concentration. Whether this effect contributes to the inhibition by these substances of the proliferation of EAT cells in vitro is still an open question.

In further experiments, it could be shown that the *O*-alkyl-glycerophosphocholine analogs 1, 2 and 6 (Table 2, C) are inhibitors of PKC, the activation of which is thought to be a prerequisite for the induction of cell proliferation. The ID_{50} of the most effective compound 2-*O*-hexadecyl-1-*O*-(2,2,2-trifluorethyl)glycero-3-phosphocholine (compound 6, Table 2, C) was about 42 μM. The corresponding values for the other compounds tested were of the same order of magnitude (data not shown). This ability to inhibit PKC also might contribute to the cytostatic action of the ALP analogs studied. The inhibitory action of ET-18-OCH_3 on PKC of leukemia cells and cultured human cell lines (HL 60, K 62) recently has been demonstrated by Helfman et al. (18).

Effects of halogen-containing lipid analogs in vivo. Some of the halogen-containing monoalkyl- and monoacylglycerol analogs investigated were found to have a distinct cytostatic activity on EAT cells in vivo (12). They are active only when in direct contact with the tumor cells, e.g. after ip application in ascites tumor-bearing animals. They hardly are active when given

TABLE 4

Efficiency of 6-Carboxyfluorescein (CF) Entrapment in Different Liposomes Made of Synthetic Alkyllysophospholipids (Chlorodeoxyhexadecylglycerophosphocholine Isomers 1-3) or Phosphatidylcholines and Cholesterol (CH)

Type	Composition[a, b]	CF Entrapment (%)
MLV[d]	1 + CH	3.5
MLV	2 + CH	2.9
MLV	3 + CH	3.1
MLV	EPC[c] + CH	2.5
MLV	HEPC[c] + CH	3.0
SUV[d]	1 + CH	1.2
SUV	2 + CH	1.1
SUV	3 + CH	0.9
SUV	EPC + CH	0.2
SUV	HEPC + CH	0.7

Isomer			
1	O-R	Cl	O-PC
2	Cl	O-PC	O-R
3	Cl	O-R	O-PC

[a]Lipid/CH molar ratio 1:1.
[b]Starting lipid concentration 40 μM.
[c]Hydrogenated (HEPC), normal egg phosphatidylcholine (EPC).
[d]Multilamellar vesicles (MLV), small unilamellar vesicles (SUV).

TABLE 5

Effectivity of Alkyl Lysophospholipid Analogs 1 and 2[a] Free or Liposomally Encapsulated in the Lewis Lung Carcinoma

Group	Substance	Application	Dose (mg/kg/day)	MDST[b] (day)	T/C[c] (%)	Body diff. 1-4 days (%)
A	comp. 2, free	po	0.5	16.0	114[d]	+15
B	comp. 1, free	po	0.5	17.0	121[d]	+11
C	SUV(comp. 2:CH 1:1)	ip	5.0	16.0	114[d]	+15
D	SUV(comp. 2:CH 1:1)	ip	0.5	17.0	121[d]	+12
E	SUV(comp. 1:CH 1:1)	ip	5.0	16.0	114[d]	+13
F	SUV(comp. 1:CH 1:1)	ip	0.5	16.5	118	+14
G	Physiol. saline	ip		14.0		+7

[a]1 = 2-Chloro-2-deoxy-1-*O*-hexadecylglycero-3-phosphocholine; 2 = 1-Chloro-1-deoxy-3-*O*-hexadecylglycero-2-phosphocholine.
[b]Median survival time.
[c]Relation of survival time of treated to control animals in %.
[d]Significance (p = 0.05) vs. control group.

subcutaneously or by another route, although the doses applied in these cases theortically should yield a sufficient level of the drug. It follows that the compounds are either degraded, poorly transported or lose their proper dispersion state. The latter apparently is important for the activity, because they only are active in vitro when applied in aqueous emulsions, and they are inactive when given in a clear solution of dimethylsulphoxide.

We also examined the efficiency of selected ALP analogs (1-3, Table 2, C) on murine ascitic P 388 leukemia and Lewis lung carcinoma. In order to compare the influence of different dispersion states, we used the substances in free form and as liposomes.

It was found that in the presence of cholesterol (CH) in 1:1 molar ratio, the isomeric chlorodeoxy-*O*-hexadecylglycerophosphocholines 1-3 form liposomes when the lipid-cholesterol mixture is hydrated. This was demonstrated by electron microscopy (not shown) and also by the entrapment of aqueous solutions containing 6-carboxyfluorescein (CF). The efficiency of entrapment of CF in liposomes (MLV, SUV) formed from the synthetic alkyl lysophospholipids, hydrogenated (HEPC) and normal egg phosphatidylcholine (EPC) is shown in Table 4.

Surprisingly, the entrapment for the water-soluble CF is much more efficient when using alkyl lysophospholipids for encapulation. Further studies show that SUV made of synthetic ALP analogs 1-3 (Table 2) are stable over 25 days, while those made of HEPC or EPC (also with cholesterol in 1:1 molar ratio) released half of their entrapped 6-carboxyfluorescein within 15 days.

Table 5 shows the results obtained with Lewis lung carcinoma in mice. Treatment with the synthetic alkyl lysophospholipids (free drug or as empty liposomes) results in a moderate life prolongation. In each case, the smaller concentrations produced better effects. No toxicity was observed. In spite of the fact that in another experiment large vesicles like MLV preferentially were taken up by lung tissues, no favorable therapeutic activity resulted in the treatment of lung metastases after surgical removal of the primary tumor (data not shown). In the leukemia P 388 of different treatment schedules (ip; op; 0.5 or 5 mg/kg/d), no differences in the T/C values in comparison to the control group were observed.

Similar results were obtained in our experiments with the compound ET-18-OCH_3. There is a noteworthy discrepancy between the strong cytostatic activity found in vitro and the low efficiency in vivo; there also is discrepancy with data reported on a significant antineoplastic activity in special tumor models (23), which should be elucidated by further experiments. It has been shown by others that the effects of phospholipid analogs on animal tumors in vivo is not as striking as the one of other known cytostatic compounds, e.g. alkylating agents or antimetabolites of the nucleic acid metabolism, and that they exert a "soft" effect. However, because their mode of action is completely different from that of the classical cytostatica, they still may be of great value in clinical practice either in combination with other agents or alone (e.g. in treatment intervals).

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Cytotoxic Effects of Ether Lipids and Derivatives in Human Nonneoplastic Bone Marrow Cells and Leukemic Cells In Vitro[1]

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The effects of 2-lysophosphatidylcholine (2-LPC), the alkyl lysophospholipid derivatives (ALP) 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH$_3$) and 1-*O*-hexadecyl-*sn*-glycero-3-phospho-trimethyl-ammonio-hexanol, the 2-acetamide analog of platelet-activating factor (PAF) 1-*O*-octadecyl-2-acetamide-*sn*-glycero-3-phosphocholine, the thioether lysophospholipid derivative (TLP) BM 41.440 and the ether-linked lipoidal amine CP-46,665 on tritiated thymidine uptake and trypan blue dye exclusion were tested in vitro in various freshly explanted cell samples from human nonneoplastic bone marrow and human leukemias. In both assay systems, a dose range of 1–20 μg/ml of the compounds was tested after 24, 48 and 72 hr of coincubation with the cells.

The trypan blue dye exclusion revealed statistically significant preferential cytotoxicity in leukemic cells for three compounds with the order of quantitative selectiveness: ET-18-OCH$_3$ > BM41.440 > 2-acetamide analog of PAF. CP-46,665 was the most toxic compound, but did not reveal significant differences between nonneoplastic bone marrow and leukemic cells when added in concentrations greater than 1 μg/ml. The trimethyl-ammonio-hexanol compound showed only minor activity in the majority of tests, when added at concentrations < 20 μg/ml. 2-LPC was rather ineffective.

The tritiated thymidine uptake showed only preferential antiproliferative effects towards leukemic cells of ET-18-OCH$_3$ and, sometimes, within the dose time frame tested of BM 41.440. All compounds tested except 2-LPC and the trimethyl-ammonio-hexanol compound were active also in this assay (inhibition of uptake >50% of the controls). Based on these results, ET-18-OCH$_3$ and BM 41.440 are recommended for experimental bone marrow purging.

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Recently there has been increasing interest in the question of selective antineoplastic cytotoxicity of ether lipids. The conflicting data accumulated so far have been reviewed (1). Interestingly, Vogler et al. have observed remarkable purging activity of the alkyl lysophospholipid derivative (ALP) ET-18-OCH$_3$ in investigating syngeneic bone marrow transplantation with a simulated remission bone marrow of the WEHI-3-B-leukemia in a mouse model in vivo (2). However, the same laboratory could not show antileukemic selectivity of ET-18-OCH$_3$ in in vitro testing of thymidine uptake and clonogenicity of human leukemic cells and normal human bone marrow cells (3). In this report we have addressed the question of antileukemic selectivity, testing the influence of a variety of different ether lipids and derivatives on thymidine uptake and trypan blue dye exclusion in vitro of nonneoplastic bone marrow cells and leukemic cells from humans.

MATERIALS AND METHODS

Drugs. The chemical structures of the lipids tested are depicted in Figure 1. 2-Lysophosphatidylcholine (2-LPC;

FIG. 1. Chemical structures of 2-LPC (A), ET-18-OCH$_3$ (B), 1-*O*-hexadecyl-*sn*-glycero-3-phospho-trimethyl-ammonio-hexanol (C), 1-*O*-octadecyl-2-acetamide-*sn*-glycero-3-phosphocholine (D), BM 41.440 (E) and CP-46,665 (F).

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Abbreviations: ALL, acute lymphocytic leukemia(s); ALP, alkyl lysophospholipid derivative(s); ANLL, acute non-lymphocytic leukemia(s); AUL, acute undifferentiated leukemia(s); CML/BC, chronic myeloid leukemia/blast crisis; FCS, fetal calf serum; 2-LPC, 2-lysophosphatidylcholine; PAF, platelet activating factor; TLP, thioether lysophospholipid derivative(s).

A) was purchased from C. Roth (Karlsruhe, FRG). The ALP ET-18-OCH_3 (B) was purchased from Medmark Chemicals (Gruenwald bei Muenchen, FRG). The 1-*O*-hexadecyl-*sn*-glycero-3-phosphotrimethyl-ammoniohexanol (C) was synthesized by one of our group. The 2-acetamide-analog of platelet-activating factor (PAF) 1-*O*-octadecyl-2-acetamide-*sn*-glycero-3-phosphocholine (D) was supplied by J. Hajdu, Department of Chemistry, California State University (Northridge). The thioether lysophospholipid derivative (TLP) BM 41.440 (E) was synthesized by Boehringer Mannheim GmbH (Mannheim, FRG). CP-46,665 (F) was supplied by K. E. Jensen, Pfizer Central Research (Groton, CT).

Cells. Cells of 36 bone marrow samples from patients without neoplastic bone marrow disease and cells of 30 patients with different types of leukemias (14 acute nonlymphocytic leukemias [ANLL], 11 acute lymphocytic leukemias/acute undifferentiated leukemias [ALL/AUL], 5 chronic myeloid leukemia/blast crisis [CML/BC]) were tested. Bone marrow samples were taken from patients who were referred for diagnostic bone marrow puncture for diseases other than solid tumors or hematologic malignancies, e.g., with anemia. The cells from the patients with the leukemias were collected predominantly from venous blood but in a few cases from the bone marrow. Informed consent of every patient was obligatory before the blood or bone marrow samples were taken for experiments. Venous blood or bone marrow was diluted with Roswell Park Memorial Institute (RPMI) 1640 medium (1:2), subsequently layered over a density gradient and spun at 400 × g for 30 min. The isolated fractions from the leukemic samples consisted morphologically of ≥95% of leukemic blasts with ≤5% of lymphocytes. The isolated fractions from the bone marrow samples contained a high percentage of granulopoietic cells, predominantly precursor cells including some band forms. In addition, these samples contained erythropoietic precursors, including normoblasts, and rarely lymphopoietic and reticulohistiocytic cell forms.

Tests. The trypan blue dye exclusion and the [^{3}H]-thymidine uptake test were performed as described before (4). In both assay systems, the cells were coincubated with the test compounds at a final concentration of 1, 5, 10 and 20 μg lipid/ml in 10% fetal calf serum (FCS)-containing medium for 24, 48 and 72 hr. Cytotoxic and cytostatic/antiproliferative activity of the lipids was measured as a decrease in trypan blue dye exclusion and an inhibition of tritiated thymidine uptake into the nuclear fraction of the cells given as percentage of the controls.

Statistical methods. The statistical comparison of the cytostatic-antiproliferative and/or cytotoxic activity of the test compounds against leukemias and bone marrow was performed by the U-test of Wilcoxon, Mann and Whitney (two-tailed).

RESULTS AND DISCUSSION

Viability of the cells based on trypan blue dye exclusion before testing was >90%. Relaible results in [^{3}H]thymidine uptake test and trypan blue dye exclusion were found in all 30 leukemias and 33 of 36 bone marrows. Three bone marrows could not be evaluated because of sterility

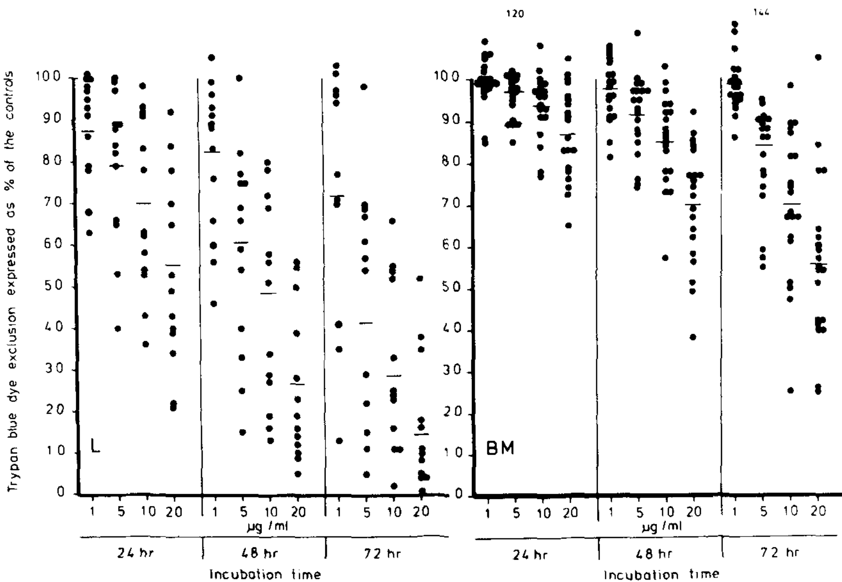

FIG. 2. Trypan blue dye exclusion of cells from non-neoplastic bone marrows (BM) and leukemias (L) from human origin after different times (24, 48 and 72 hr) of incubation with ET-18-OCH_3 at 1, 5, 10 and 20 μg/ml. The y axis gives the viability values (trypan blue dye exclusion) expressed as percentage of the controls. The x axis shows two blocs, results obtained for L on the left and BM on the right. Each block gives three compartments of values, one for a single incubation time as indicated. Each compartment contains four vertical columns of dots representing the results. Each dot and each arabic number represent the results of one patient's sample. Bars represent means of results obtained with one certain concentration of the compound at one incubation time as indicated. The significance levels from the comparisons between the cytotoxicity in L and BM cells are given for each concentration and incubation time (columns) in Table 1.

problems during culture. Furthermore, cell numbers were limited in some of the samples obtained. Thus, it was not possible to test every substance in every experiment. However, the results of all experiments performed are given here without exception, and the actual number of samples tested with each single compound can be observed in the figures.

Trypan blue dye exclusion. The cytotoxicity of the compounds as measured in the trypan blue dye exclusion test is depicted in the scattergrams of Figures 2–6. Figure 2 visualizes a higher cytotoxicity of ET-18-OCH_3 on the leukemic blast populations of various donors than on the nonneoplastic bone marrow cell samples of various donors. The viability based on trypan blue dye exclusion of the leukemic cell samples decreases with lower concentrations and shorter incubation times with the drug than the viability of the nonneoplastic cell samples. Comparable findings were made testing BM 41.440 (see Fig. 3) and the 2-acetamide analog of PAF (see Fig. 4). CP-46,665 clearly was the most toxic substance (see Fig. 5). Its LC_{50} value (the lethal concentration for 50% of the cells; mean of all tested samples) after 24 hr of incubation with leukemic cells was ≤5 μg/ml. The order of cytotoxic efficacy in leukemias was CP-46,665 > ET-18-OCH_3 ≥

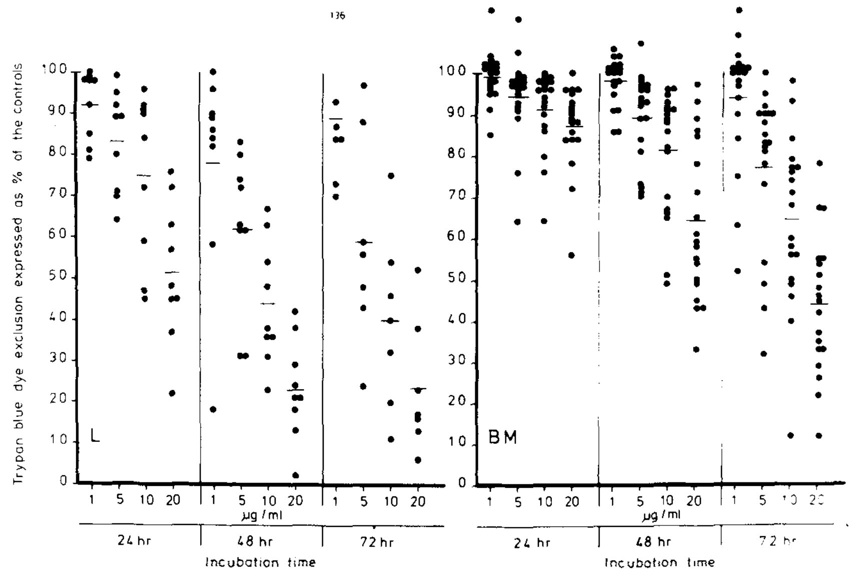

FIG. 3. Trypan blue dye exclusion of non-neoplastic bone marrow and leukemic cell populations after incubation with BM 41.440. For details see legend to Fig. 2.

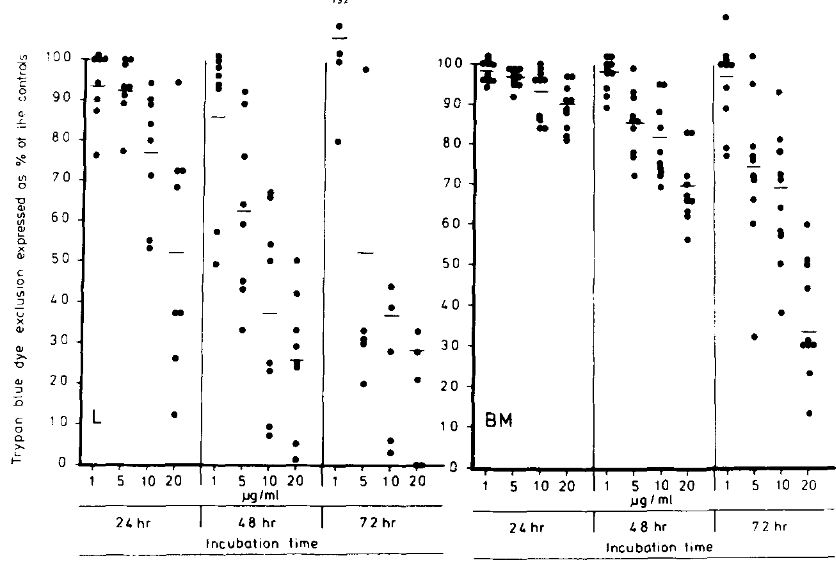

FIG. 4. Trypan blue dye exclusion of nonneoplastic bone marrow and leukemic cell populations after incubation with the 2-acetamide analog of platelet-activating factor 1-*O*-octadecyl-2-acetamide-*sn*-glycero-3-phosphocholine. For details see legend to Fig. 2.

BM 41.440 ⩾ 1-*O*-octadecyl-2-acetamide-*sn*-glycero-3-phosphocholine. These compounds revealed a clear dose- and time-dependent relationship in their cytotoxicity profiles. 1-*O*-Hexadecyl-*sn*-glycero-3-phosphotrimethyl-ammonio-hexanol (see Fig. 6) failed to show considerable cytotoxicity and reached an LC_{50} only at ⩾20 μg/ml, after more than 48 hr of incubation with the leukemic cells. 2-LPC, which was tested as a reference for detergent-like surface activity, remained without major effects within the dose and time schedule tested (details not shown). All six compounds were compared in μg/ml because of their similar molecular weight.

Statistical comparison of the viability in trypan blue dye exclusion test of all bone marrow samples with all leukemic samples after incubation with the ether-lipid derivatives demonstrated significant quantitative selectivity of the cytotoxicity of three compounds in leukemic cells (see Table 1). In this evaluation (Table 1) each p-value represents the significance level from the comparison of the according data pairs (columns) in Figures 2–6. The order of selectivity among these three lipids was ET-18-OCH_3 > BM 41.440 > 2-acetamide analog of PAF. However, there was considerable variability in the sensitivity of both bone marrow samples and leukemic

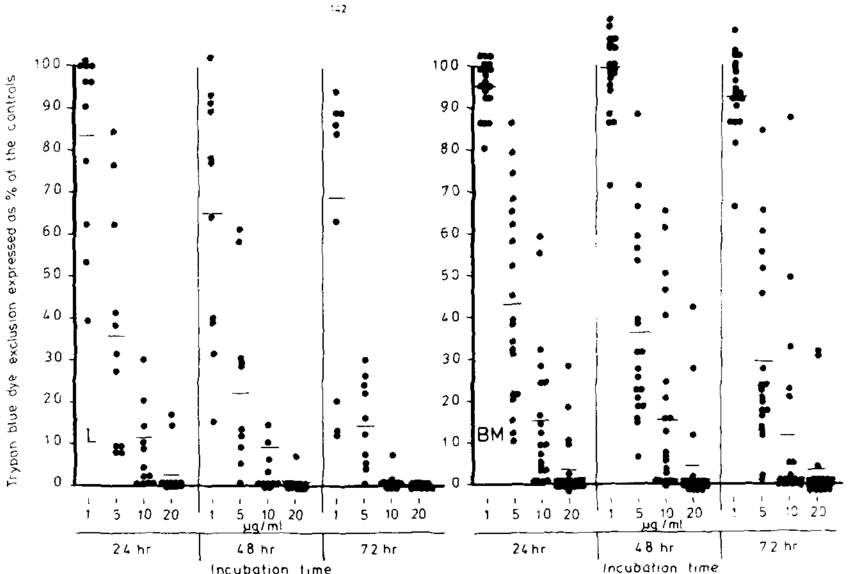

FIG. 5. Trypan blue dye exclusion of nonneoplastic bone marrow and leukemic cell populations after incubation with CP-46,665. For details see legend to Fig. 2.

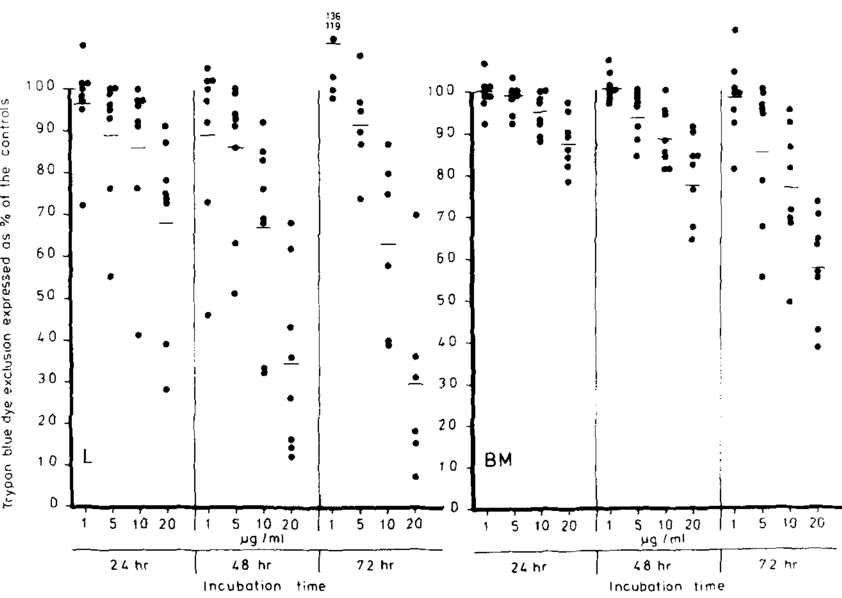

FIG. 6. Trypan blue dye exclusion of nonneoplastic bone marrow and leukemic cell populations after incubation with 1-*O*-hexadecyl-*sn*-glycero-3-phospho-trimethyl-ammonio-hexanol. For details see legend to Fig. 2.

samples (see figures), excluding qualitative or complete selectiveness of the drugs. Furthermore, whereas an incubation of 48 hr was better than 24 and 72 hr to show this antileukemic selectivity, it was not possible to determine an optimal concentration between 1 and 20 μg/ml. CP-46,665 failed to demonstrate considerable antileukemic selectivity when added in concentrations higher than 1 μg/ml, and the selectivity pattern of the trimethyl-ammonio-hexanol compound is questionable because of its low activity in the dose range tested.

Tritiated thymidine uptake. Figure 7 depicts the effects of ET-18-OCH_3 on thymidine uptake of leukemic and non-neoplastic bone marrow cells. There was a high variability among the various donor samples with regard to sensitivity. After 48 hr of incubation, 5 and 10 μg ET-18-OCH_3 per ml seemed to have selective preferential antiproliferative effects on leukemic cells in this assay (see Fig. 7 and Table 1). With few exceptions (e.g., BM 41.440; 5 μg/ml after 48 hr and ≥ 10 μg/ml after 72 hr), the scattergrams and statistical analyses of all other compounds tested revealed no selective antiproliferative effects on leukemic cells (details not shown). However, all substances tested except 2-LPC and the trimethyl-ammonio-hexanol compound were also active in this assay and reached LC_{50}-values at concentrations of ≤10 μg/ml and with ≥48 hr of incubation. The activity and putative selectivity of the trimethyl-ammonio-hexanol compound can only be judged after incubation in a higher dose range.

In both assays, our results indicated neither a higher sensitivity of bone marrows over leukemias nor significant differences in the sensitivity of ALL, AUL, ANLL or CML/BC. However, we have included leukemia patients with relapses after previous chemotherapy in this series, which may be an explanation for the variable sensitivity observed.

These data contradict previous in vitro results reported by Vogler et al. (3), who did not find selective antileukemic activity of ET-18-OCH_3 testing tritiated thymidine uptake and clonogenicity of leukemic cells from patients in comparison with normal bone marrow samples. However, these authors discussed in detail that a substantial percentage of the leukemic cell populations failed to grow colonies under their test conditions and that the plating efficiency was low. We had the same experience with freshly explanted leukemias irrespective of conditioned media, since ca. 1/3 of the cell samples did not grow colonies, which might introduce selection bias. Thus, we have concluded that clonogenicity might not be the exclusively

TABLE 1

Significance Levels of the Selective Activity of Various Ether-Lipid Derivatives on Leukemia Cells in Comparison with Bone Marrow Cells In Vitro[a]

Lipid	Concentration (μg/ml)	P-values 24 hr	P-values 48 hr	P-values 72 hr
ET-18-OCH_3[b]	1	0.0074	0.0056	0.0343
	5	0.0022	0.0003	0.0009
	10	0.0011	0.000	0.0001
	20	0.0003	0.000	0.000
	1	n.s.[c]	n.s.	n.s.
	5	0.0057	0.0035	n.s.
	10	n.s.	0.0234	n.s.
	20	n.s.	n.s.	n.s.
BM 41.440	1	0.0261	0.0011	n.s.
	5	0.0137	0.0006	n.s.
	10	0.009	0.0002	0.0119
	20	0.0001	0.000	0.0192
1-*O*-octadecyl-2-acetamide-*sn*-glycero-3-phosphocholine	1	n.s.	n.s.	n.s.
	5	n.s.	0.0312	n.s.
	10	0.018	0.0008	n.s.
	20	0.0052	0.0007	n.s.
CP-46,665	1	n.s.	0.0007	0.0325
	5	n.s.	n.s.	n.s.
	10	n.s.	0.0394	n.s.
	20	n.s.	n.s.	n.s.
1-*O*-hexadecyl-*sn*-glycero-3-phosphotrimethyl-ammonio-hexanol	1	n.s.	n.s.	n.s.
	5	n.s.	n.s.	n.s.
	10	n.s.	0.033	n.s.
	20	0.033	0.0045	0.0300

[a]U-test of Wilcoxon, Mann and Whitney (see Materials and Methods).

[b]For ET-18-OCH_3, the P-values for both trypan blue dye exclusion test (above) and tritiated thymidine uptake (below) are depicted. For all other test compounds only the values for trypan blue dye exclusion test are given.

[c]Not significant.

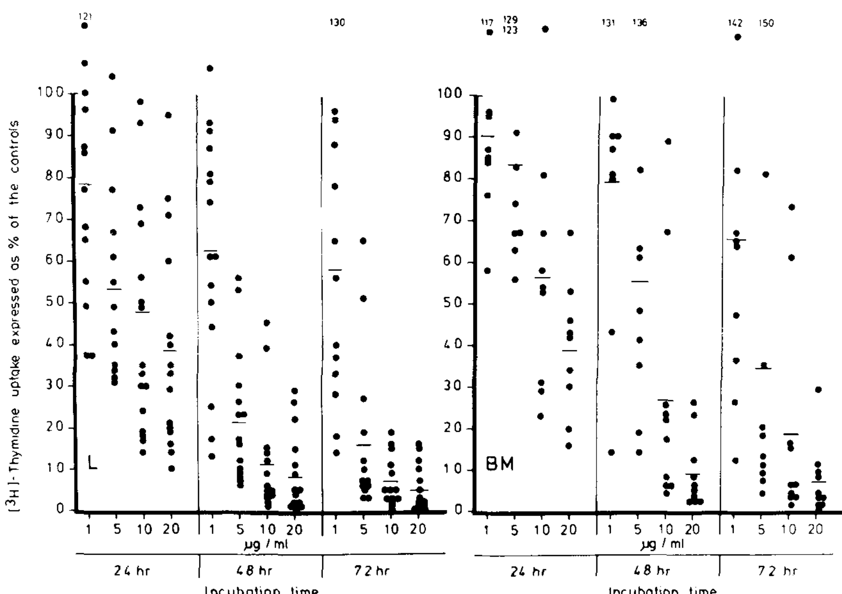

FIG. 7. Uptake of tritiated thymidine of cells from nonneoplastic bone marrows (BM) and leukemias (L) from human origin after different times (24, 48 and 72 hr) of incubation with ET-18-OCH$_3$ at 1, 5, 10 and 20 μg/ml. Each dot and each arabic number represent the mean of triplicates of one patient's sample. Bars represent means of the samples tested at the same incubation time and with the same concentration.

appropriate technique to answer statistically the question of quantitative selectivity. On the other hand, since ether-lipid derivatives are known to produce membrane damage (1,4–7), this being their major direct mode of action, an assay system measuring single cell viability and membrane damage as the trypan blue dye exclusion test seems also appropriate. In addition, previous experiments have shown a good correlation of trypan blue dye exclusion assay and clonogenicity for ether lipids in leukemic cell lines and cells of solid tumors (4). Furthermore, our data are in accordance with the in vivo experiments of Vogler's group (2) as mentioned above, which are well reflected by our in vitro findings.

The reason for the differences between [^{3}H]thymidine uptake and trypan blue dye exclusion remains speculative. However, in our hands [^{3}H]thymidine uptake is very sensitive, already detecting sublethal cell injury.

In addition, our results regarding the preferential antineoplastic cytotoxicity of these types of ether-lipid derivatives are in accordance with the results of others comparing various virus-transformed and leukemic cell lines of murine and human origin with different types of normal cells (1,8–12). Malignant cell lines, however, may represent an artificially sensitive target, and adult neutrophils or skin fibroblasts (10) do not reflect adequately the toxicity pattern of cytotoxic chemotherapy. Thus, our study is a necessary prerequisite for both experimental procedures for autologous bone marrow transplantation in leukemic patients and further drug design, with the aim of finding lipid derivatives with a better therapeutical index. The order of quantitative selectivity that we have found (ET-18-OCH$_3$ > BM 41.440 > 2-acetamide analog of PAF) indicates a recommendation in favor of the first two structures for bone marrow purging. However, this study has not been designed to find effective purging concentrations and times for the lipids for leukemic blasts or safe concentrations and times for survival of BM cells, since this can be better addressed in an experimental in vivo study observing, for example, syngeneic bone marrow "take" and survival in rodents.

Finally, if among the various modes of actions by which ether-lipid derivatives influence neoplastic cells either via mediators or directly (for further discussion see ref. 1), the direct membrane-based cytotoxicity plays a dominant role for therapeutic activity, then it seems reasonable to examine the potential value of lipid structures designed to exploit metabolic differences between normal and neoplastic tissue by our in vitro approach. In this respect, earlier papers have emphasized the O-alkyl-cleavage enzyme (12–14). To further test this hypothesis, it might be necessary to add higher concentrations of the "tailor-made" trimethyl-ammonio-hexanol compound to check on the selectivity theoretically warranted in an active dose range. This compound can be metabolized by the O-alkyl-cleavage enzyme, but not by phospholipases, and thus might selectively accumulate in cells that lack this enzyme. However, various other enzymes within lipid metabolism should be examined and metabolites of phospholipase C deserve more attention (1,15,16).

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Structure-Cytotoxicity Studies on Alkyl Lysophospholipids and Some Analogs in Leukemic Blasts of Human Origin In Vitro[1]

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Eleven lipids have been tested for cytotoxic (trypan blue dye exclusion) activity in cells from eight freshly explanted human leukemias in vitro. 4-Aminomethyl-1-[2,3-(di-N-decyloxy)*N*-propyl]-4-phenylpiperidine (CP-46,665), 1-mercapto-hexadecyl-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440), the 2-acetamide analog of platelet-activating factor (PAF) and 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH$_3$) were found among the most active compounds. 2-Lysophosphatidylcholine (2-LPC) showed the lowest activity. However, in addition there was variation among the results regarding the activity of the 1-octadecyl-*rac*-glycero-3-phosphocholine (ET-18-OH) and its D- and L-forms, but a significantly higher cytotoxic activity of D-ET-18-OH compared with L-ET-18-OH on the basis of 2-LPC as control after an incubation time of 48 hr. We conclude that with the limited number of structures available, this type of study is not sufficient to yield further information about the mode of the accumulation and toxicity of this type of lipids.
Lipids 22, **911–915 (1987).**

During recent years, there has been increasing interest in the antitumor activity of certain ether lipids and their derivatives (1). Among various modes of action, some alkyl lysophospholipid derivatives (ALP) have shown direct effects on neoplastic cells, since they destroy leukemic (2–4) and tumor cells (5,6) during coincubation. In former studies (7), the relationship between in vitro toxicity and structure of a variety of ALP has been tested after incubation with neoplastic cells from solid human tumors and HL 60 leukemic blasts. From these studies, it was concluded that the alkyl-linkage in the *sn*-1 position of the molecule is a necessary prerequisite for cytotoxicity. Furthermore, in the majority of tumors tested the substitution of the *sn*-2 position is necessary for cytotoxicity.

The aim of the study, which is summarized in this communication, was to learn more about structure-toxicity relationship concerning the cytotoxic (trypan blue dye exclusion) activity of various ether lipid derivatives in cells from freshly explanted human leukemias.

MATERIALS AND METHODS

Drugs. Eleven lipids have been tested for cytotoxic activity in human leukemic blasts in vitro. In detail, the following substances have been studied: (A) the ether-linked lipoidal amine 4-aminomethyl-1-[2,3-(di-N-decyloxy)*N*-propyl]-4-phenylpiperidine (CP-46,665) (provided by K. E. Jensen, Pfizer Central Research, Groton, CT, (B) the thioether-lysophospholipid derivative 1-mercapto-hexadecyl-2-methoxymethyl-*rac*-glycero-3-phosphocholine BM 41.440 (Boehringer Mannheim GmbH, D-6800 Mannheim, FRG), (C) the 2-acetamide-analog of platelet-activating factor (PAF) 1-*O*-octadecyl-2-acetamide-*sn*-glycero-3-phosphocholine (synthesized and provided by J. Hajdu, Department of Chemistry, California State University, Northridge, CA) (8), (D) the 1-*O*-hexadecyl-*sn*-glycero-3-phospho-trimethyl-ammonio-hexanol (synthesized by one of our group), the alkyl lysophospholipid derivatives (E) 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH$_3$) (Medmark Chemicals, D-8022 Gruenwald bei Muenchen, FRG), (F) 1-*O*-octadecylpropane-3-phosphocholine (ET-18-H), (G) 1-*O*-dodecylpropanediol-3-phosphocholine (ET-12-H), (H) 1-*O*-octadecyl-*rac*-glycero-3-phosphocholine (ET-18-OH) (provided by P. G. Munder, Max Planck-Institute for Immunobiology, D-7800 Freiburg, FRG), (I) D-ET-18-OH and (J) L-ET-18-OH (synthesized by one of our group) and (K) the ester-linked 2-lysophosphatidylcholine (2-LPC) (Fa. Roth, D-7500 Karlsruhe, FRG). The chemical structures are given in Figure 1.

Cells. Cells of eight freshly explanted human leukemias (four ALL, three ANLL, one CML/BC) were tested. The leukemic blasts were obtained by density gradient preparation of venous blood or bone marrow aspirate. The isolated fractions consisted of $\geq$95% of leukemic blasts with $\leq$5% of lymphocytes. Viability of the cells based on trypan blue dye exclusion before testing was $\geq$90%.

Trypan blue dye exclusion was performed by a method described recently (9). In this assay system, 5×10^5 cells/ml were coincubated with the test compounds at a final concentration of 1, 5, 10 and 20 μg lipid/ml 10% serum-containing medium for 24, 48 and 72 hr. Cytotoxic activity of the tested lipids was measured as a decrease in trypan blue dye exclusion given as percentage of the controls. Comparison of simultaneous evaluations by different individuals revealed a reproducibility of the method within a range of $\leq$10%.

Statistical analysis. The values for trypan blue dye exclusion have been examined in the Wilcoxon matched-pairs, signed-ranks test (2-tailed).

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Abbreviations: ALP, alkyl lysophospholipid derivative(s); 2-LPC, 2-lysophosphatidylcholine; ANLL, acute non-lymphoblastic leukemia; ALL, acute lymphoblastic leukemia; CML/BC, chronic myeloid leukemia/blast crisis; PAF, platelet-activating factor; CP-46,665, 4-aminomethyl-1-[2,3-(di-N-decyloxy)*N*-propyl]-4-phenylpiperidine; BM 41.440, *rac*-1-mercapto-hexadecyl-2-methoxymethyl-*rac*-glycero-3-phosphocholine; ET-18-OH, 1-*O*-octadecyl-*rac*-glycero-3-phosphocholine; ET-18-OCH$_3$, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ET-18-H, 1-*O*-octadecylpropane-3-phosphocholine; ET-12-H, 1-*O*-dodecylpropanediol-3-phosphocholine.

FIG. 1. Chemical structures of the various lipids being tested for cytotoxic activity in human leukemic blasts in vitro. A, CP-46,665; B, BM 41,440; C, 2-acetamide analog; D, trimethyl-ammoniohexanol compound; E, ET-18-OCH_3; F, ET-18-H; G, ET-12-H; H (I, J), ET-18-OH (I, D-form; J, L-form); K, 2-LPC.

RESULTS AND DISCUSSION

Data concerning trypan blue dye exclusion of the leukemic blasts under the influence of the various lipids are summarized in Table 1. In most of the examined leukemias, loss of the ability of trypan blue dye exclusion proved dependent on dosage of the drug and incubation time. 2-LPC showed only minor activity in all of the eight leukemias in the dose range of 1–20 μg/ml. In one case resistant to previous chemotherapy (ANLL 1), the leukemic blasts revealed low sensitivity to 10 of the 11 lipids. The only effective compound was CP-46,665, which had a complete cytotoxic activity after 48 hr of incubation. CP-46,665 showed the highest efficacy in all examined leukemias after at least 24 hr of drug exposure.

For the other lipids, except 2-LPC and CP-46,665, there was variation among the results regarding their cytotoxic activity in the different leukemias. To rank the tested compounds concerning their cytotoxic activity, the values of each compound were compared statistically to the values of each other at each dose level and incubation time (Wilcoxon test). Because of similar molecular weight, all compounds were compared in μg/ml. Resulting p-values are summarized in Table 2, in which as one example the values after an incubation time of 48 hr are shown. Interpretation of the cytotoxicity-relationship between the compounds starting on the x-axis reveal (A) CP-46,665, (B) BM 41.440, and (C) the 2-acetamide analog as being the most cytotoxic drugs with significantly higher activity than most of the other lipids.

However, when Table 2 is evaluated starting with the y-axis the (A) lipoidal amine CP-46,665 does not meet any other drug with significantly higher cytotoxicity, which is not true for (B) BM 41.440 and (C) the 2-acetamide analog. Thus, data indicate (A) CP-46,665 as being the structure with the highest cytotoxic activity. (B) BM 41.440, (C) 2-acetamide analog and (E) ET-18-OCH_3 follow in this order of activity when evaluated starting on the x- and the y-axis, since only (A) CP-46,665 shows significantly higher cytotoxicity. (K) 2-LPC shows the lowest activity. Evaluation of significance levels at other incubation times did not add more information to this study (data not shown).

There was variation among the results regarding the cytotoxicity of the (H) *rac* ET-18-OH and its (I, J) D- and L-forms. In contrast to the results obtained with HL 60 leukemic cell line (7) in which the D-form of ET-18-OH showed higher activity than the L-form, we have found in freshly explanted leukemias four cell samples (ANLL 1, 3; ALL 2, 3) in which D- and L-forms were rather ineffective and four cell samples (CML/BC; ANLL 2; ALL 1,4) in which both isomeres had nearly the same cytotoxicity. Table 2 shows no significant p-values between (H) *rac*-, (I) D- and (J) L-ET-18-OH in direct comparison at the same dose levels after 48 hr of incubation time, which was also true for the other times of incubation. (I) D-ET-18-OH, on the other hand, showed a significant effect at 20 μg/ml after 48 hr compared with (K) 2-LPC at 20 μg/ml after 48 hr ($p = 0.04$).

To obtain more information, Figure 2 shows the significant differences of each compound, compared with 2-LPC as the reference compound for direct detergent-like activity at each dose level after 48 hr. CP-46,665, BM 41.440 and the 2-acetamide-analog are again the most active compounds. Regarding the *rac* ET-18-OH and its D- and L-isomers, D-ET-18-OH showed a higher activity than the L-form and the racemate, as the *rac* ET-18-OH and the L-ET-18-OH showed a lower number of significant values than the D-form. Evaluation of significance levels at each dose level after 48 hr also has been done for each

TABLE 1

Influence of Various Lipids on the Viability (Trypan Blue Dye Exclusion) of Cells from Freshly Explanted Human Leukemias

Leukemia type	Incubation time (hr)	Concentration (μg/ml)	Trypan blue dye exclusion expressed as percentage of the controls for given compounds A*	B	C	D	E	F	G	H	I	J	K
ANLL[a] 1	24	1	102	98	100	100	100	101	99	97	97	100	100
		5	96	98	94	100	100	95	98	97	98	100	98
		10	67	96	92	97	97	92	96	98	99	99	97
		20	2	85	92	92	92	94	82	94	89	99	97
	48	1	88	89	99	96	96	96	100	100	98	99	99
		5	82	82	86	96	94	93	96	100	93	95	101
		10	42	78	79	88	68	86	78	101	96	99	96
		20	0	71	76	82	63	69	76	101	95	89	87
	72	1	99	101	91	101	91	104	91	104	97	104	100
		5	76	96	86	103	88	70	94	97	94	97	91
		10	26	97	87	80	90	96	93	97	91	93	91
		20	0	78	76	83	80	78	54	80	76	78	88
ANLL 2	24	1	100	98	100	101	100	100	100	100	101	100	100
		5	84	99	100	93	97	100	98	99	98	98	99
		10	20	91	89	97	92	100	97	95	96	97	100
		20	0	57	68	75	65	88	79	89	82	89	91
	48	1	78	86	94	92	88	97	96	107	97	93	99
		5	61	83	76	86	75	92	83	81	83	90	90
		10	6	54	50	95	56	85	86	69	85	90	89
		20	0	13	29	14	39	50	21	31	39	33	71
	72	1	89	87	80	119	96	80	52	67	115	113	-
		5	30	48	30	74	61	46	50	46	104	102	93
		10	0	46	28	39	24	37	37	37	74	41	93
		20	0	13	28	7	35	20	0	17	29	26	76
ANLL 3	24	1	100	100	94	98	100	100	100	101	99	95	100
		5	76	95	100	100	99	100	100	96	91	100	100
		10	0	96	94	96	98	97	100	98	97	99	100
		20	0	63	94	91	92	95	96	94	100	95	95
	48	1	102	90	98	100	96	94	99	97	101	107	92
		5	58	80	89	93	100	104	100	101	94	106	86
		10	0	48	66	92	80	67	92	96	84	109	98
		20	0	18	42	43	28	51	58	89	69	86	93
	72	1	102	136	132	112	150	100	156	94	148	156	140
		5	22	88	98	108	98	122	128	100	-	144	136
		10	0	44	44	80	54	-	138	96	140	122	140
		20	0	38	21	36	16	30	102	96	114	110	144
CML/BC[b]	24	1	85	96	-	86	122	82	75	75	82	81	83
		5	88	100	-	78	115	80	69	58	90	72	81
		10	52	60	-	102	78	71	-	60	75	74	75
		20	20	43	-	82	58	63	-	54	56	53	49
	48	1	88	96	-	77	90	73	96	83	98	79	98
		5	50	82	-	94	102	85	100	85	98	92	110
		10	39	42	-	79	67	65	92	85	98	73	79
		20	17	39	-	63	25	18	88	54	52	50	49
	72	1	76	66	-	82	126	108	126	53	74	66	79
		5	39	63	-	53	113	102	108	50	110	-	87
		10	8	45	-	66	59	49	74	34	74	68	95
		20	0	29	-	76	29	39	47	9	42	15	50
ALL[c] 1	24	1	102	99	101	101	93	91	89	100	74	90	99
		5	20	89	93	100	91	95	90	101	91	79	93
		10	0	90	80	100	85	84	63	79	80	70	100
		20	0	48	72	74	56	61	51	66	71	-	77
	48	1	72	58	57	73	100	78	60	80	73	-	78
		5	32	63	50	63	75	68	68	53	73	75	57
		10	0	53	45	68	93	68	65	73	40	48	78
		20	0	38	23	62	106	35	50	72	38	47	80
	72	1	77	70	113	136	104	57	45	75	98	93	86
		5	32	59	-	95	91	48	41	59	64	47	-
		10	0	75	-	75	89	39	45	70	55	48	66
		20	0	22	-	70	107	3	25	45	29	30	45
ALL 2	24	1	-	91	90	95	91	95	91	-	95	84	94
		5	-	89	89	95	91	86	91	-	94	87	88
		10	-	84	84	91	76	81	80	-	86	87	92
		20	-	72	72	87	59	71	57	-	79	70	89
	48	1	-	100	93	102	90	111	114	-	91	101	107
		5	-	62	59	94	76	91	88	-	88	93	100
		10	-	38	54	69	56	75	71	-	97	82	90
		20	-	29	24	68	37	32	53	-	78	-	99
	72	1	-	84	109	103	77	97	111	-	98	98	98
		5	-	43	33	97	57	72	87	-	90	104	95
		10	-	20	39	87	33	46	74	-	90	93	105
		20	-	16	33	31	26	26	25	-	84	-	75
ALL 3	24	1	101	100	101	101	100	100	96	99	100	100	100
		5	8	97	94	100	93	97	98	97	100	99	100
		10	0	94	92	98	78	89	100	100	97	99	99
		20	0	90	86	74	77	87	98	96	98	100	99
	48	1	72	110	114	95	113	102	89	91	101	90	97
		5	0	100	87	105	70	-	101	107	108	103	100
		10	0	86	74	103	66	83	105	110	103	102	106
		20	0	55	53	74	49	60	99	92	95	92	105
	72	1	68	145	137	103	134	105	94	106	109	111	109
		5	0	55	86	134	69	95	105	114	128	122	102
		10	0	30	25	123	35	72	125	119	126	100	119
		20	0	24	16	-	11	27	86	111	91	114	111
ALL 4	24	1	76	81	76	72	86	94	89	90	85	65	106
		5	27	71	77	55	82	74	53	64	81	72	91
		10	0	45	53	41	62	65	45	67	73	56	87
		20	0	22	26	28	21	44	60	58	69	38	59
	48	1	31	68	49	46	105	79	60	68	54	64	85
		5	0	31	43	51	69	59	43	-	-	61	84
		10	0	23	25	33	51	50	60	66	54	59	80
		20	0	16	25	16	29	49	43	62	18	29	28

[a] ANLL, acute nonlymphoblastic leukemia; [b] CML/BC, chronic myeloid leukemia/blast crisis; [c] ALL, acute lymphoblastic leukemia

* A, CP-46,665; B, BM 41.440; C, 2-acetamide-analog; D, 1-O-hexadecyl-sn-glycero-3-phospho-trimethyl-ammonio-hexanol; E, ET-18-OCH$_3$; F, ET-18-H; G, ET-12-H; H, racemic ET-18-OH; I, D-ET-18-OH; J, L-ET-18-OH; K, 2-LPC

TABLE 2
Significant P-values (Wilcoxon Test) for Each Compound Compared to Each Other Concerning Their Cytotoxic Activity After an Incubation Time of 48 hr at the Same Dose Levels

Compounds	Concentration (µg/ml)	A	B	C	D	E	F	G	H	I	J	K
K	20	p=0,02	p=0,01	p=0,02	p=0,04	p=0,07	p=0,03			p=0,04		
	10	p=0,02	p=0,01	p=0,02	p=0,02	p=0,03	p=0,01	p=0,06				
	5	p=0,02	p=0,04	p=0,03								
	1	p=0,05	p=0,08		p=0,09							
J	20	p=0,02	p=0,02	p=0,03								
	10	p=0,02	p=0,02	p=0,02								
	5	p=0,02	p=0,01	p=0,02			p=0,06					
	1	p=0,08										
I	20	p=0,02	p=0,02	p=0,03								
	10	p=0,02	p=0,04	p=0,03								
	5	p=0,03	p=0,03	p=0,03								
	1	p=0,04										
H	20	p=0,02	p=0,02	p=0,03	p=0,03		p=0,04					
	10	p=0,02	p=0,02	p=0,03			p=0,08					
	5	p=0,03		p=0,04								
	1	p=0,06			p=0,08							
G	20	p=0,02	p=0,01	p=0,05								
	10	p=0,02	p=0,02	p=0,03		p=0,09+						
	5	p=0,02	p=0,02	p=0,03								
	1											
F	20	p=0,02		p=0,04								
	10	p=0,02	p=0,02	p=0,02								
	5	p=0,03	p=0,02	p=0,03								
	1											
E	20	p=0,02										
	10	p=0,02	p=0,09									
	5	p=0,02										
	1	p=0,04										
D	20	p=0,02	p=0,02									
	10	p=0,02	p=0,01	p=0,02								
	5	p=0,02	p=0,02	p=0,02								
	1											
C	20	p=0,03										
	10	p=0,03										
	5	p=0,03										
	1											
B	20	p=0,02										
	10	p=0,02										
	5	p=0,03*										
	1											
A	20											
	10											
	5											
	1											

*p-values < 0.05 indicate that the compound on the x-axis shows a significantly higher cytotoxic effect than the compound on the y-axis at the same dose level.
p-values < 0.05 for compounds on the y-axis indicate, however, that this compound has a significantly lower cytotoxic activity than the compound on the x-axis at the same dose level.

+p-values between 0.05 and 0.10 are given to show tendencies of borderline significance.

A=CP-46,665; B=BM 41.440; C=2-acetamide analog; D=1-O-hexadecyl-*sn*-glycero-3-phospho-trimethyl-ammonio-hexanol; E=ET-18-OCH_3; F=ET-18-H; H=racemic ET-18-OH; I=D-ET-18-OH; J=L-ET-18-OH; K=2-LPC.

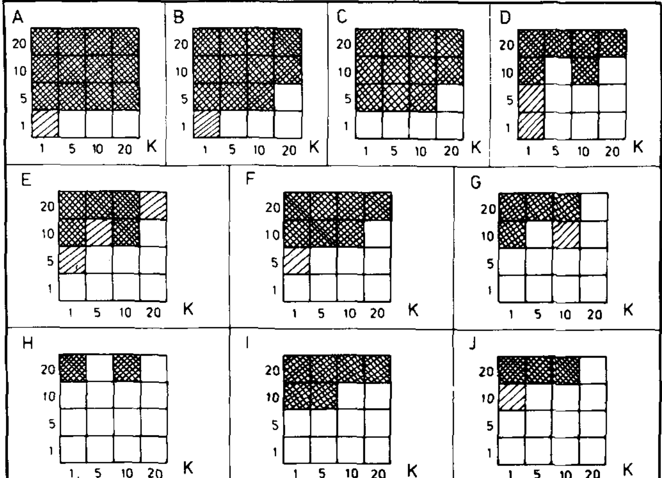

FIG. 2. Significant p-values for each compound concerning their cytotoxic activity compared with 2-LPC as reference drug at each dose level after an incubation time of 48 hr. Hatched box, $p < 0.05$; which indicates a significantly higher cytotoxic activity of the compound on the y-axis compared with (K) 2-LPC at the different dose levels (1, 5, 10 and 20 μg/ml) after an incubation time of 48 hr. Lined box, $0.05 \leqslant p < 0.10$; which indicates a borderline significance concerning the cytotoxic activity of the compound on the y-axis compared with (K) 2-LPC at the different dose levels (1, 5, 10 and 20 μg/ml) after an incubation time of 48 hr. Open box, no significant p-value ($p \geqslant 0.10$).

compound that is compared with another but did not add further information.

Trypan blue dye exclusion showed a correlation with the corresponding values obtained from [^{3}H]-thymidine uptake as another test system (data not shown). However, thymidine uptake was a more sensitive assay system showing toxic effects of the lipids on a lower dose level and after shorter incubation times. Furthermore, in comparison to other series (2,4,9) the results presented here with a group of patients resistant to or relapsing after previous chemotherapy showed comparably poor toxity of all tested lipids, which underlines the variability of the cytotoxicity when freshly explanted cells from patients in contrast to cell lines are tested (Schich et al., this issue of *Lipids*).

We conclude from our data that there is remarkable individual variation among different freshly explanted human leukemias concerning their sensitivity to the cytotoxic effect of glycerol-backbone lipid derivatives. The compounds analyzed for structure-activity relationships have shown the necessity of a metabolically stable *sn*-1 position for activity, and that activity increases with stable *sn*-2 and *sn*-3 positions. However, with the limited number of structures available, this type of study is not sufficient to yield additional information about the mode of accumulation and toxicity of this type of lipid.

ACKNOWLEDGMENT

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Antineoplastic Activity of the Thioether Lysophospholipid Derivative BM 41.440 In Vitro[1]

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Thioether lysophospholipid derivatives (TLP) inhibited the in vitro uptake of [^{3}H]thymidine into blasts of eight leukemias and cells of 12 different solid tumors of human origin. This effect correlated with trypan blue dye exclusion, which was used to assess cell damage.

Cytostatic and cytotoxic effects of TLP were dependent on dosage and incubation time. Destruction of leukemic blasts was completed with >5 μg/ml after an incubation of >48 hr, but 10 to 20 μg/ml were necessary in solid tumors. Ester-linked 2-lysophosphatidylcholine was ineffective in the same dose range, which points to the requirement of the alkyl moiety in *sn*-1 and a stable *sn*-2 substitution of the molecule for the antineoplastic effect.

To assess putative antileukemic selectivity, the cytotoxicity (trypan blue dye exclusion) of TLP was compared in human cell samples of 19 non-neoplastic bone marrows and 9 leukemias. Results revealed a significantly higher activity of the TLP BM 41.440 in leukemic blasts.

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Thioether lysophospholipid derivatives (TLP) are analogs of the naturally occurring 2-lysophosphatidylcholine (2-LPC), which is an important intermediate in the phospholipid metabolism of cell membranes (1). Analogs of 2-LPC (e.g., the alkyl lysophospholipid derivative [ALP] ET-18-OCH_3) have previously been shown to possess antitumor activity. They destroy leukemic (2,3) and tumor (4,5) cells in vitro, inhibit the growth (6,7) and metastasis (8) of syngeneic murine tumors and have been used successfully in treating experimental rat tumors (9).

This activity is suggested to be partially mediated by enhancing the cytotoxicity of macrophages (8) and direct effects on neoplastic cells, such as disturbance of the phospholipid metabolism (10) and inhibition of protein kinase C (11). However, induction of cell differentiation of leukemic blasts (12) and antiinvasiveness (13) are also discussed as possible modes of action.

TLP have been synthesized in order to obtain a new class of antitumor lipids with increased metabolic stability and possible higher antineoplastic effects than the analogs investigated earlier. First results on the cytotoxicity of TLP have been previously published (14). In this report, we summarize our experiments on cytotoxicity of TLP in human leukemic blasts, some solid tumors and on antileukemic selectivity of BM 41.440.

MATERIALS AND METHODS

TLP derivatives. Five different TLP derivatives were synthesized by Boehringer Mannheim GmbH (Mannheim, FRG) and tested in comparison with the ALP ET-18-OCH_3 and the ester-linked 2-LPC. The chemical structure of BM 41.440 (1-hexadecyl-mercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine) is given in Figure 1.

Cells. Blasts of eight leukemias (four acute leukemias [AL], four blast crises of chronic myelogenous leukemia [CML/BC]) and cells of 12 different neoplastic cell lines of human origin (four non-Hodgkin's lymphomas of T- and B-cell origin, six hypernephromas, one medulloblastoma, one glioblastoma) were incubated with various concentrations (1–20 μg/ml) of the test compounds for 24–72 hr. To measure possible selective antileukemic cytotoxicity of BM 41.440, cell samples of 19 non-neoplastic human bone marrows and nine human leukemias were compared.

Test systems. Two test systems were used to assess the antiproliferative and cytotoxic effects. In the [^{3}H]thymidine uptake, the antiproliferative action of the lipids was measured as a decrease of [^{3}H]thymidine uptake into the cells. The trypan blue dye exclusion test was used to identify the cytotoxic effects of the test substances.

Statistical analysis. The Friedman two-way analysis and the U-test according to Wilcoxon, Mann and Whitney were used.

Details on the characterization, isolation and cultivation of the cells tested and the test systems used have been published before (14,15).

RESULTS AND DISCUSSION

In the first set of experiments, five different TLP and the ALP ET-18-OCH_3 were tested, comparing their cytostatic and antiproliferative effects in various leukemias and solid tumors. No striking difference in efficacy could be observed between the test substances. All analogs significantly reduced the proliferation rates of the cells in a time- and dose-dependent way. Incubation with

$H_2C-S-(CH_2)_{15}-CH_3$

$HC-CH_2-O-CH_3$

$H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

FIG. 1. Chemical structure of the thioether lysophospholipid derivative BM 41.440.

[1]Presented at the symposium on "Ether Lipids in Oncology," Göttingen, Federal Republic of Germany, December 1986.

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Abbreviations: TLP, thioether lysophospholipid derivative(s); ALP, alkyl lysophospholipid derivative(s); 2-LPC, 2-lysophosphatidylcholine; AL, acute leukemia(s); CML/BC, blast crisis of chronic myelogenous leukemia.

>5 μg/ml (>10 μg/ml in solid tumors) resulted in a complete inhibition of proliferation in the cell types tested. For further studies, we concentrated on BM 41.440. Figure 2 displays the cytostatic effect of BM 41.440 in cells of four AL and four CML/BC. More than 24 hr of incubation and a concentration of at least 5 μg/ml were necessary to reveal full antineoplastic activity. 2-LPC, tested in the same dose range, was ineffective or showed only minor action (data not shown).

In 12 cell lines from lymphomas (4×) and solid tumors (one glioblastoma, one medulloblastoma, six hypernephromas) of human origin, up to 20 μg/ml of BM 41.440 were required to produce proliferation rates around 10% of the controls. Except for one medulloblastoma, all cell lines were sensitive to the test compound in a time- and dose-dependent way (details not shown).

To assure that incubation with TLP not only decreased proliferation of neoplastic cells as measured in [³H]-thymidine uptake assay, but actually caused cell death, the trypan blue dye exclusion test was performed. A correlation was found between viability, expressed as

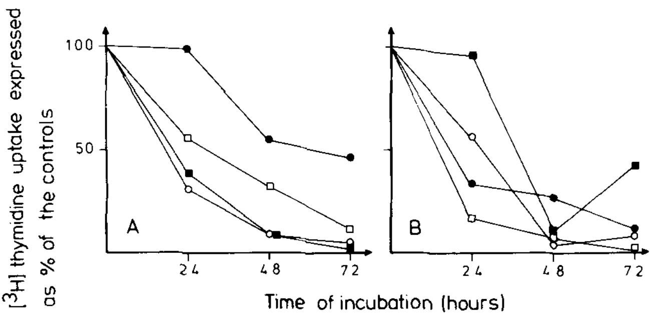
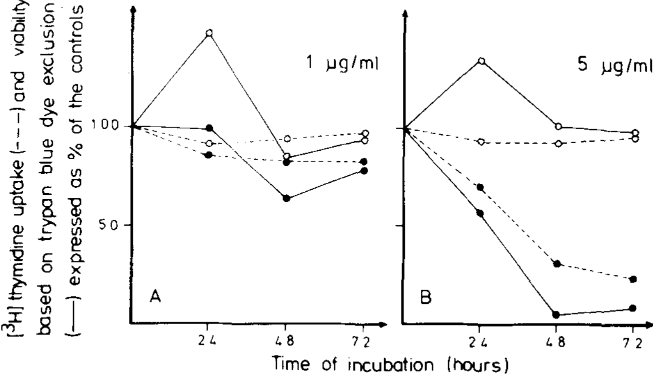


FIG. 2. (A) [³H]Thymidine uptake of cells of four different acute leukemias after incubation with 5 μg/ml BM 41.440 for the times indicated. (B) [³H]Thymidine uptake of cells of four different blast crises of chronic myelogenous leukemias after incubation with 5 μg/ml BM 41.440 for the times indicated.



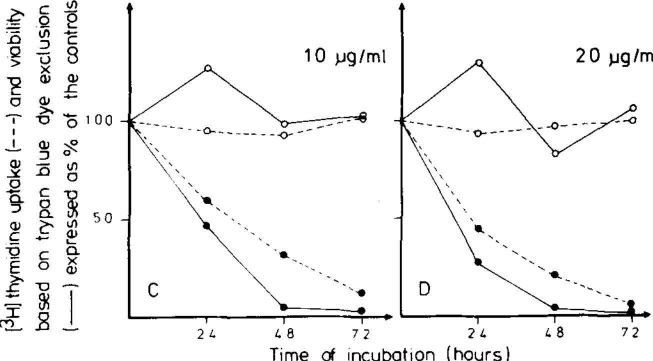


FIG. 3. [³H]Thymidine uptake (— — —) and viability based on trypan blue dye exclusion (———) of a blast crisis of chronic myelogenous leukemia after 24, 48 and 72 hr of incubation with 2-lysophosphatidylcholine (O) and BM 41.440 (●).

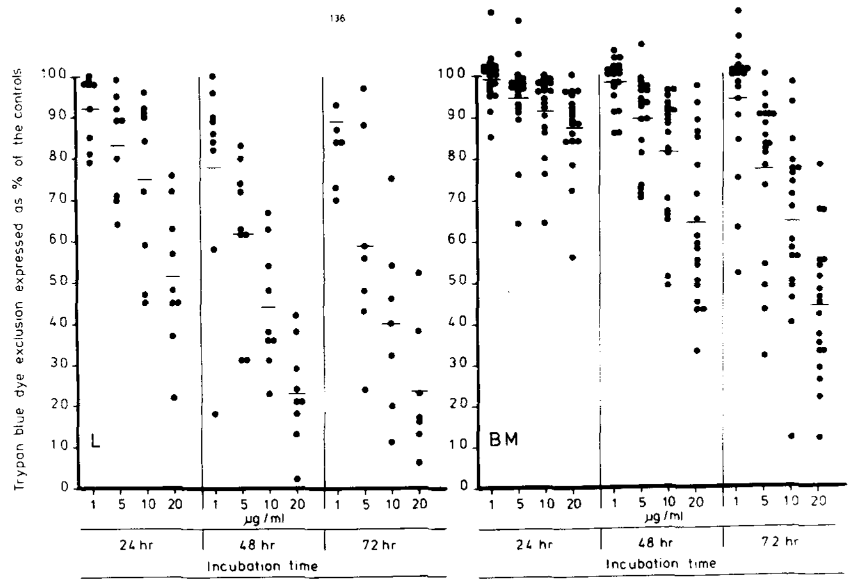


FIG. 4. Trypan blue dye exclusion of leukemias (L) and non-neoplastic bone marrows (BM) after incubation with BM 41.440 at the times indicated. Each dot and each arabic numeral represent one patient's sample. Bars represent mean values of all samples at a given dose and time.

TABLE 1

Significance Levels of the Selective Activity of BM 41.440 in Human Leukemia Cells Compared with Non-neoplastic Bone Marrow Cells In Vitro[a]

Concentration of BM 41.440 (μg/ml)	P-value at time of incubation		
	24 hr	48 hr	72 hr
1	0.0261	0.0011	n.s.[b]
5	0.0137	0.0006	n.s.
10	0.009	0.0002	0.0119
20	0.0001	0.0	0.0192

[a]U-test of Wilcoxon, Mann and Whitney (see Materials and Methods).

[b]n.s., Not significant.

percent of the controls, and inhibition of [^{3}H]thymidine uptake in leukemic blasts and, with few exceptions, in cell lines of solid tumors. Dependency on dosage and incubation time as well as inactivity of 2-LPC was confirmed by the trypan blue dye exclusion in the tested dose range. However, trypan blue dye exclusion proved to be less sensitive than thymidine uptake. Examples are given in Figure 3. This figure furthermore shows that after incubation times of more than 48 hr, a saturation of the cytotoxic/cytostatic properties of BM 41.440 could be reached. Previous studies on the destructive effects of TLP on outer cell membranes, as visualized by scanning electron microscopy (14), indicate membrane destruction as an important factor in the cytotoxicity of these compounds.

Possible selective antileukemic cytotoxicity of BM 41.440 was assessed by comparing the results of the trypan blue dye exclusion in human cell samples of 19 non-neoplastic bone marrows and nine leukemias, including patients with relapses after previous chemotherapy. Significantly higher activity of BM 41.440 was found in leukemic blasts, again in a time- and dose-dependent way. Results are given in Figure 4 and Table 1. Table 1 depicts the statistical processing of the results given in Figure 4.

Results indicate that TLP, as a new group of ether-lipid derivatives, exert strong cytostatic and cytotoxic activity in vitro in cells of human leukemias and solid tumors in a time- and dose-dependent way. Five μg/ml or more of the analog BM 41.440 completely inhibited the [^{3}H]-thymidine uptake into cells of leukemic blasts after an incubation time of more than 24 hr; 10–20 μg/ml were necessary in solid tumors. Ester-linked 2-LPC proved to be ineffective in the same dose range; thus with due caution the alkyl moiety in *sn*-1 and a stable *sn*-2 substitution of the molecule seem to be required for the antineoplastic effect.

Results of cytotoxic activity in human leukemic blasts compared with non-neoplastic bone marrow cells indicate a significantly higher activity of BM 41.440 in leukemic blasts, which recommends this compound for experimental bone marrow purging, including further in vitro and in vivo research. However, based on the study outlined here and other in vivo studies, clinical phase I trials with BM 41.440 are underway (16).

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Experimental Studies on the Role of Alkyl Lysophospholipids in Autologous Bone Marrow Transplantation[1]

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The selective cytocidal effect of alkyl lysophospholipids against neoplastic cells while sparing normal cells make these ideal candidates for purging leukemic cells from bone marrows obtained during remission. To test the feasibility of such an approach, a murine model and an in vitro human cell model were developed. In the murine system a mixture of normal bone marrow cells and WEHI IIIB myelomonocytic leukemic cells was incubated with varying doses of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-Me) for 24 hr before being injected into tail veins of lethally irradiated Balb/c mice. At doses of 20 and 100 μg/ml, long-term survivors were noted. The additonal steps of freezing and thawing following incubation resulted in significantly longer survival with doses of 10 to 50 μg/ml, but were toxic to marrow stem cells at 100 μg/ml.

In the in vitro model, normal marrow progenitor cells and leukemic cells (the promyelocytic cell line HL60) were exposed to varying concentrations of ET-Me for 1 and 4 hr alone or mixed, and clonogenicity was assayed by colony formation in semisolid medium during 7–14 days' incubation. At doses up to 100 μg/ml exposed for 4 hr normal progenitor cells were spared and HL60 colonies eliminated. Other phospholipids analogues were less effective in eliminating leukemic cells, but spared normal progenitor cells.

A survey of fresh leukemic cells found varying degrees of sensitivity to ET-Me, indicating the need for testing a variety of compounds.

These studies clearly indicated the potential usefulness of alkyl lysophospholipid compounds in selectively purging leukemic cells from remission marrows for autologous bone marrow transplantation.
Lipids 22, 919–924 (1987).

Alkyl-lysophospholipids (ALP) have been reported to have selective cytocidal activity against neoplastic tissues while sparing normal cells (1–3). The mechanism of action appears to be directed against the cell membranes. If so, these compounds would be excellent candidates for treating neoplastic diseases. Some antitumor activity has been observed in laboratory animals and humans (4–9).

The reason for this selectivity has been thought to be the ability of normal tissues to metabolize the compounds through an O-alkyl cleavage enzyme that splits the fatty acid off the glycerol backbone at the *sn*-1 position. A number of neoplastic tissues have been shown to lack this enzyme, and the compound accumulates, damaging the cell membrane (10,11). However, there is evidence that some of the more active compounds are poor substrates for the cleavage enzyme, and thus some other mechanisms may be active (12,13).

Regardless of the mechanism for selectivity, these compounds warrant investigation. Although the full toxicity spectrum of these agents is unknown, in preliminary clinical trials some toxicity has been observed (9). An alternative use of these agents would be to serve as purging agents to rid remission bone marrows, obtained from patients treated for leukemia, of residual neoplastic cells, allowing the collection and cryopreservation of leukemic-free marrow for autologous transplantation. To test the feasibility of such an approach, we have developed a murine model and an in vitro human model. The results of most of these studies have been previously published and are presented here along with some new data (14–16).

METHODS

Compounds. For the in vivo experiments using the murine model, only one compound was used; 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-Me) was supplied initially by Dr. Paul Munder of the Max Planck Institute, Freiburg, FRG, and later by Dr. Wolfgang Berdel of the Technical University, Munich, FRG. A stock solution of the compound was prepared by dissolving 1 mg in 1 ml of Roswell Park Memorial Institute (RPMI) medium containing 20% fetal bovine serum (FBS) (Hazelton Dutchland Laboratories, Denver, PA) and stored at −20 C.

For the in vitro experiments, additional compounds used included the lipoidal amine (4-aminomethyl-1-[2,3-(di-n-decycloxy)-n-propyl]-4-phenylpiperidine dihydrochloride [CP] supplied by Dr. K. E. Jensen, Central Research, Pfizer, Groton, CT [17]). CP was dissolved in RPMI medium containing 10% FBS prior to use. 1-Hexadecyl-2-acetamide-deoxyglycero-phosphorylcholine (ET-Ac) was a gift from Dr. Joseph Hajdu, Northridge, CA. A stock solution of ET-Ac and ET-Me was prepared by dissolving 1 mg in 1 ml of RPMI 1640 medium containing 10% FBS and was stored at −20 C.

In all instances, the solutions were sterilized by micropore filtration (0.22 m, Gelman Sciences, Ann Arbor, MI).

Cells. For in vivo studies, WEHI IIIB leukemic cells, a myelomonocytic leukemia (18), which has been passaged in Balb/c mice in our laboratory since 1972, were obtained from ascitic fluid. Normal murine bone marrow cells (NMB) were obtained from 12-wk-old female Balb/c mice by flushing femoral shafts with RPMI medium. The cells were passaged through wire mesh (300 microns) to make single cell suspensions into medium containing 20% FBS and 1% PNS (penicillin-neomycin-streptomycin) antibiotic solution (Gibco Laboratories, Grand Island, NY). To

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Abbreviations: ALP, alkyl lysophospholipid; CFU-S, colony forming units spleen; CP, 4-aminomethyl-1-[2,3-(di-n-decycloxy)-n-propyl]-4-phenylpiperidine dihydrochloride; ET-Ac, 1-*O*-hexadecyl-2-acetamidodeoxyglycero-phosphocholine; ET-Me, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; FBS, fetal bovine serum; LC, leukemic; NBM, normal bone marrow; RPMI, Roswell Park Memorial Institute; SRM, simulated remission marrow; TBI, total body irradiation.

simulate a remission marrow (SRM), mixtures of normal marrow cells and WEHI IIIB cells were made at a ratio of 50:1.

For in vitro studies, the promyelocytic leukemia cell line HL60 (19) was obtained from Dr. Robert Gallo, National Institutes of Health, Bethesda, MD. Earlier studies had shown the line to be quite sensitive to ET-Me (20). These cells were passaged twice weekly in RPMI 1640 medium containing 10% FBS. All studies were carried out in log phase growth. NBM cells were obtained from bone fragments provided at surgery for orthopedic procedures. The fragments were crushed, suspended in supplemented McCoy's 5A medium (Gibco) and mononuclear cells separated by centrifugation over Ficoll-Hypaque (Histopaque-1077, Sigma Chemical Co., St. Louis, MO). The interface cells were washed twice with RPMI 1640 medium and resuspended in RPMI 1640 with 10% FBS. SRM was prepared by mixing normal marrow cells from male donors with HL60 cells in a ratio of 1000:1.

Cells obtained by aspiration from the posterior iliac crest at the time of diagnosis or relapse of leukemic (LC) patients who gave informed consent were used to test the spectrum of sensitivity of these compounds.

Spleen colony assay (CFU-s). All mice wre 12-wk-old female Balb/c obtained from Jackson Laboratories (Bar Harbor, ME) or from our own breeding colony. Irradiated mice received a total dose of 750 rads of total body irradiation (TBI) from ^{137}Cesium source (Gamma Cell Atomic Energy Ltd., model GC-40, Ottawa, Ontario, Canada) 24 hr before transplantation. Cell preparations were injected into the tail veins. The spleen colony assay was similar to that previously described (14). The mice were killed at eight days, spleens were removed and fixed in H_2O/glacial acetic acid/formalin/95% ethanol (5:1:1:3, v/v/v/v) and the number of macroscopic colonies on the antihilar surface was counted.

Survival. Mice not killed were followed for survival dating from the time of initiation of experiments. Fifteen to 30 mice were used for each time point. Autopsies were performed on all mice. Those surviving seven months were killed then.

Assay for in vitro colony formation. Mixed hematopoietic colonies, erythroid bursts, erythroid colonies and granulocyte-macrophage colonies and clusters were assayed by the method of Fauser and Messner (21). Clusters contained less than 40 cells. The cells were cultured in medium containing 0.8% methylcellulose, 30% FBS, 0.3% bovine serum albumin, 5×10^{-5} M mercaptoethanol, 5.6×10^{-8} M selenium, 10% normal human serum, 10% human placental conditioned medium (22), 5% phytohemagglutin in stimulated lymphocyte conditioned medium and 1 U/ml of erythropoietin (Step III, Connaught, Ontario, Canada) and were scored at 14 days.

Incubations and cryopreservation procedures. For the in vitro studies, 1-ml suspensions of 5×10^6 cells (NBM, SRM or LC) or 500–1000 HL60 cells were incubated at varying concentrations of phospholipid analogs for 1 and 4 hr at 37 C in 5% CO_2. They were washed twice in RPMI 1640 medium containing 10% FBS, either plated directly or frozen at −1 C/min to −80 C in 10% dimethyl sulfoxide and stored in the vapor phase of liquid nitrogen in freezing vials (Nunc, Denmark). Rapid thawing was accomplished by immersion of the vials in a 37 C water bath and followed by stepwise dilution of the dimethyl sulfoxide to 0.1% by medium. Deoxyribonuclease (type I, Sigma) at a final concentration of 100 μg/ml was used when there was cell clumping.

Murine cell suspensions (NBM, SRM or WEHI IIIB) containing varying concentrations of phospholipid analogs were incubated at 37 C in 5% CO_2 in freezing vials for 24 hr and were either injected directly into tail veins of lethally irradiated mice or were subjected to controlled-rate freezing as described above and cryopreserved. After a minimum of 24 hr, the cells were rapidly thawed and diluted with medium, and 8×10^4 cells were injected into tail veins of irradiated mice.

Statistical methods. The student t-test was used for comparisons of means and the Chi-square test for comparisons of enumeration data. A p value of <0.05 was considered significant.

RESULTS

Murine studies. The effect of 24-hr exposure of NBM cells to ET-Me on CFU-s is illustrated in Figure 1. As can be seen, there was only a small reduction at 20 μg/ml, indicating a sparing effect on murine stem cells. There was a 30% reduction following cryopreservation, but an adverse effect of ET-Me was not observed until doses of 100 μg/ml were reached (data not shown).

The role of ET-Me in purging LC cells from SRM is shown in Figure 2. As can be seen, doses of 2.5 and 5 μg/ml were no different from untreated SRM, and the mice died of leukemia between days 23 and 28. At 10 μg/ml there was some effect, but all mice eventually died of leukemia. At 20 and 100 μg/ml there was significant prolongation of life with 80% for 20 μg and 100% for 100 μg surviving

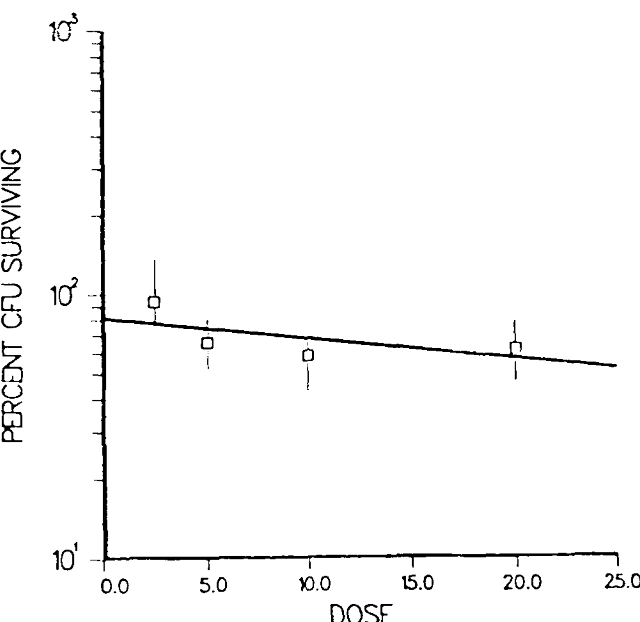

FIG. 1. Ten mice at each point were given 730 rads total body irradiation followed 24 hr later by intravenous injection of normal bone marrow cells after 24 hr of exposure in vitro to varying concentrations of ET-Me at 37 C. Macroscopic colonies were counted on the antihilar surface of spleens obtained from mice killed on day 8. Data are expressed as percentages of control ± SEM. Dose refers to μg/ml of ET-Me in the in vitro incubation mixture.

100 days. This survival is similar to mice receiving NBM and TBI. In contrast, survival of mice only irradiated without infusing NBM died within 11.5 days.

To determine if the combination of ET-Me treatment and cryopreservation would accomplish a similar result, these experiments were repeated with the additional step of freezing the cells after exposure to ET-Me. As can be seen in Table 1, doses of 10, 20 and 50 μg/ml increased the median survival time in a dose-dependent fashion and significantly increased the number of long-term survivors. However, the 100 μg/ml dose was too toxic to the stem cells after cryopreservation and thawing. These studies demonstrate the selective cytotoxic effects of ET-Me on leukemic cells while sparing NBM stem cells.

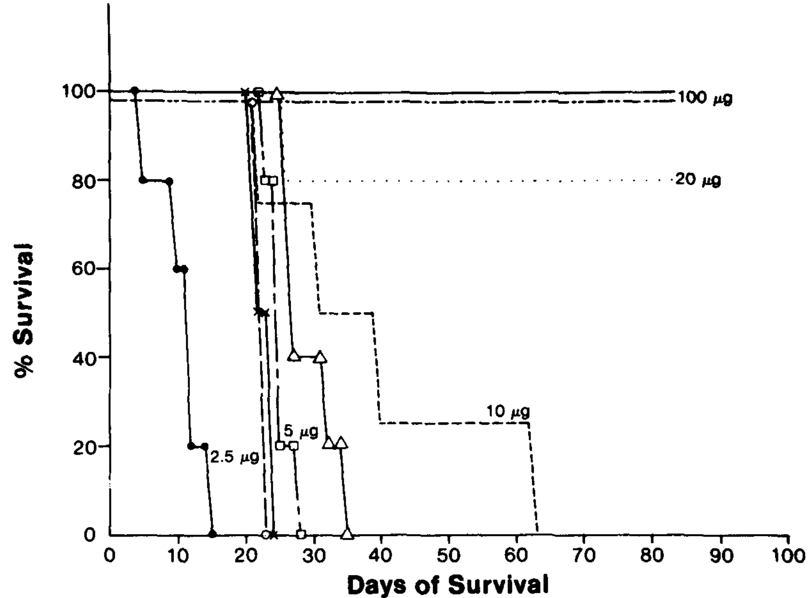

FIG. 2. Survival of Balb/c mice not transplanted or transplanted with normal bone marrow (NBM), simulated remission marrow (SRM) or SRM exposed in vitro to varying concentrations of ET-Me. ●—●, Not transplanted; ——, received NBM cells; ×—×, received SRM; ○—○, received SRM exposed to 2.5 μg/ml; □—□, received SRM exposed to 5 μg/ml; — — —, received SRM exposed to 10 μg/ml; · · · ·, received SRM exposed to 20 μg/ml; —··—··, received SRM exposed to 100 μg/ml; △—△, received SRM, not irradiated. All received 750 rads total body irradiation.

TABLE 1

Effect of Cryopreservation Following Exposure of Simulated Remission Marrow to Varying Doses of ET-Me

	Conditions[a]					Survival		
	TBI	NBM	SRM	ET-Me (μg/ml)	Number of mice	Median (days)	90 Days	p[b]
1.	+	−	−	−	30	15	1[c]	
2.	+	+	−	−	30	NR[d]	18	
3.	−	−	+	−	28	26	1[e]	
4.	+	−	+	−	29	20	9	
5.	+	−	+	5	30	20	1	NS
6.	+	−	+	10	30	25	9	<.01
7.	+	−	+	20	29	36	12	<.01
8.	+	−	+	50	15	45	4	<.02
9.	+	−	+	100	14	15	0	NS

[a]TBI, total body irradiation; NBM, cryopreserved normal murine bone marrow; SRM, cryopreserved simulated remission marrow; ET-Me, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine.

[b]p value compared to row 4. NS, Not significant.

[c]Died aplastic day 107.

[d]Not reached.

[e]Died of leukemia day 133.

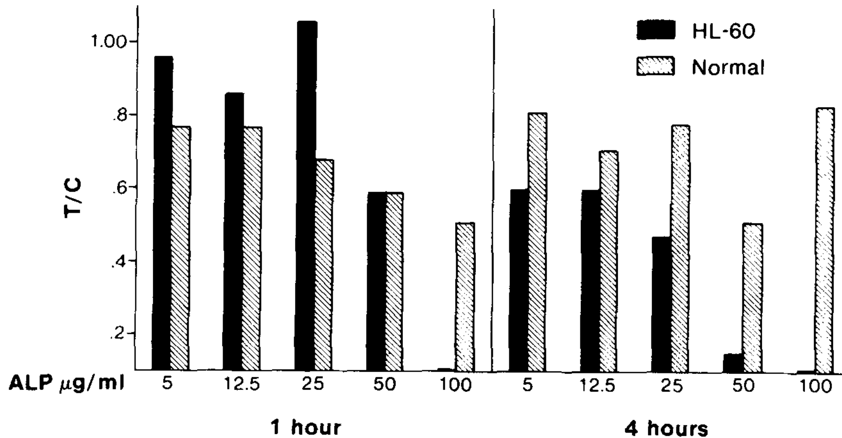

FIG. 3. The effect of alkyl lysophospholipid (ET-Me) on clonogenicity of HL60 cells and total normal bone marrow (NBM) progenitor cells. The cells were incubated for 1 or 4 hr with varying concentrations of ET-Me prior to plating. There were 500 HL60 cells plated and 10^5 NBM cells plated. Results are expressed as percentage of controls.

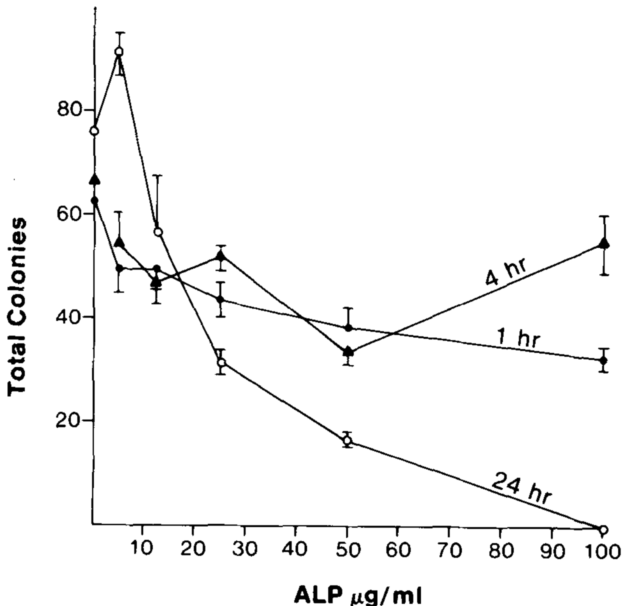

FIG. 4. The effect of alkyl lysophospholipid (ET-Me) concentration on clonogenicity of total marrow progenitor cells exposed in vitro for 1, 4 or 24 hr prior to plating. Conditions are as described in Methods. 10^5 Cells/plate were scored at 14 days. The bars are standard errors of the mean of quadriplicate samples.

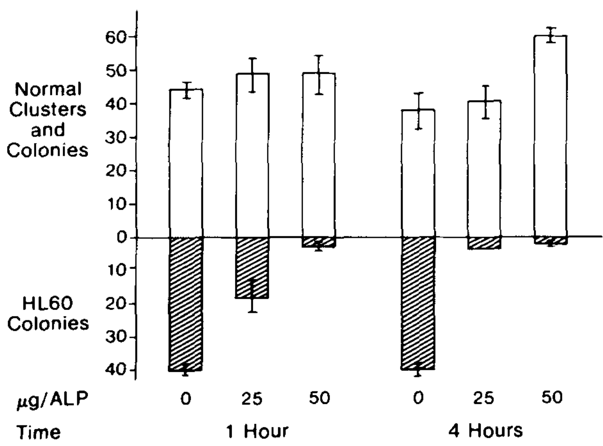

FIG. 5. The effect of alkyl lysophospholipid (ET-Me) on clonogenicity of mixed cultures of normal blood marrow (NBM) and HL60 cells. Cultures of 10^5 NBM cells and 10^2 HL60 cells were incubated with 0, 25 or 50 μg/ml of ET-Me for 1 or 4 hr. Colonies were identified by morphologic appearance.

In vitro human studies. The effect of ET-Me on clonogenicity of HL60 cells and NBM progenitors is shown in Figure 3. The HL60 cells were cultured as described above, except that erythropoietin was not added. As shown, incubation of HL60 cells for 1 or 4 hr with ET-Me resulted in a significant inhibition of colonies at 100 μg/ml and above. The effect on the total proportion of normal progenitor cells at 1 or 4 hr of incubation was minor at doses up to 100 μg/ml. However, as shown in Figure 4, there was a progressive dose-related inhibition when cells were incubated for 24 hr before cloning. Figure 5 demonstrates the purging effect of ET-Me using mixtures of HL60 cells and NBM progenitors. The colonies were identified morphologically.

These studies were repeated after cryopreservation and thawing. Between 60 and 70% of the prefreeze normal colonies could be recovered after freezing and thawing, and no dose effect of ET-Me could be observed. HL60 cells were not significantly reduced in the controls following freezing and thawing, but were further reduced by cryopreservation after exposure to ET-Me. Exposure to 50 μg/ml of ET-Me for 4 hr followed by freezing and thawing reduced HL60 colonies to zero. Thus, the combination of ET-Me and cryopreservation resulted in enhanced killing of HL60 cells while preserving 60–70% of the progenitor cells.

Effect of CP and ET-Ac on HL60 and NBM cells. Table 2 compares the effect of the compounds on colony formation by NBM cells and HL60 cells. As can be seen, neither CP nor ET-Ac was as selectively cytotoxic to HL60 cells as ET-Me. Other compounds are under development and will need to be tested in a similar fashion.

TABLE 2

Comparative Effects of Phospholipid Analogs on Clonogenicity of HL60 and Normal Bone Marrow (NBM) Progenitors

1 hr	Compounds (μg/ml)	Percent of control		
		ET-Me	CP	ET-Ac
HL60	0	100	100	100
	25	39.0 ± 0	57.5 ± 2.1	66.1 ± 0.1
	50	10.8 ± 3.1	31.8 ± 5.6	30.5 ± 0.9
NBM	0	100	100	100
	25	—	—	95.9 ± 1.6
	50	98.8 ± 1.3	88.6 ± 2.9	96.5 ± 6.0

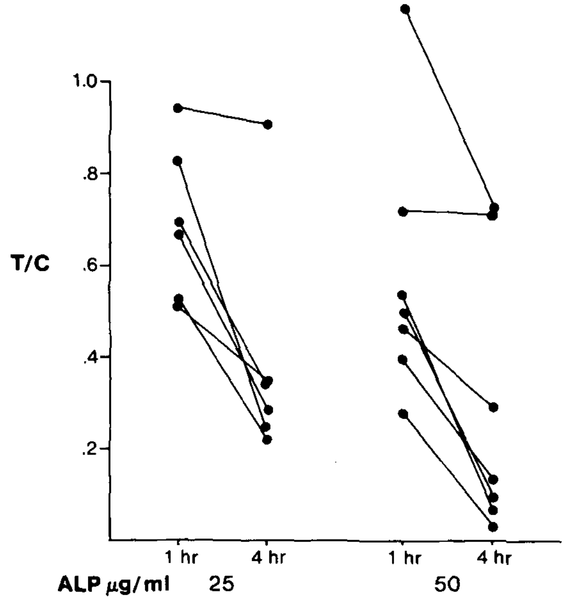

FIG. 6. The effect of alkyl lysophospholipid (ET-Me) on clonogenicity of leukemic cells obtained from patients at diagnosis or relapse expressed as ratios of treated to control values. Cells were incubated with 0, 25 or 50 μg/ml of ET-Me for 1 and 4 hr at 37 C prior to plating. Colonies were counted on day 14.

Clinical survey. Figure 6 illustrates the effects of ET-Ac on colony formation by cells obtained from LC patients at diagnosis or relapse. A wide spectrum of activity was present, and a dose effect was observed. The mean inhibition after incubation at 50 μg/ml was 50% at 1 hr and 31% at 4 hr. However, as shown, cells from some individuals were exquisitely sensitive. Other phospholipid analogs have not been tested in this fashion.

DISCUSSION

These experiments clearly demonstrate that phospholipid analogs are active antileukemic compounds and that some degree of selectivity is apparent. NBM stem cells in the mouse and NBM progenitor cells in humans appear to be much less sensitive to these compounds than neoplastic cells. The biochemical mechanisms for this selectivity have not been fully elucidated, and more studies are needed. Furthermore, an indirect antitumor effect as elicited by macrophages exposed to these analogs (23) was not studied in this series of experiments.

We attempted to demonstrate selectivity and to develop a practical, clinically feasible approach utilizing short incubation times with suitable doses and assessing the added effect of cryopreservation. It appears that the conditions necessary to initiate a clinical study have largely been established.

A clinical survey indicated that there was variability in sensitivity to ET-Me among patients. Therefore, it would be important to test the relative efficacy of a series of these compounds against a spectrum of leukemic cells as well as test the sensitivity of a particular patient's cells to a number of compounds. In this way, the most active agents to use could be defined for the individual patient for subsequent purging.

In conclusion, these studies have demonstrated the relative selective antitumor activity of three compounds. ET-Me appears to be the most effective and conditions for its use have been established to initiate clinical studies in bone marrow purging. Nevertheless, variability has been demonstrated in the sensitivity of leukemic cells obtained from patients and more of these compounds should be tested against a wider spectrum of leukemic cells and progenitor cells to find those that are most promising.

ACKNOWLEDGMENT

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Distribution and Metabolism of Hexadecylphosphocholine in Mice[1]

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Distribution and metabolic fate of radiolabeled hexadecylphosphocholine (He-PC) has been studied in mice. It is demonstrated that He-PC is well-absorbed from the intestinal tract, intravenous (IV) and oral administration lead to similar distributions throughout the body, the highest accumulation of radioactivity occurs in liver, lung and kidney, and the metabolic products are radioactive choline, phosphocholine and 1,2-diacylphosphatidylcholine. The occurrence of these metabolites indicates that phospholipases C and D may be involved in He-PC breakdown.

Lipids 22, 925–926 (1987).

Alkylphosphocholines (APC) represent a new class of antineoplastic agents with remarkable antitumoral activity in vitro (1) and in vivo (2). APC have been developed in studies on the structure-activity relationship of alkyl lysophospholipids (ALP) and possibly represent the minimal structural requirement of ALP. It was the aim of this study to analyze the biodistribution of hexadecylphosphocholine (He-PC) as a representative compound of APC. In addition, initial investigations were carried out on the in vivo metabolism of He-PC in mouse liver.

MATERIAL AND METHODS

Hexadecylphospho-[^{3}H]choline was obtained by methylation of the corresponding hexadecylphosphoethanolamine with [^{3}H]methyl iodide (3); He-PC was synthesized as earlier described (4,5).

Biodistribution. Female NMRI mice (25 to 30 g) were used in this study. Twenty-five μg of He-PC (specific activity: 50 μCi/μmol) was dissolved in 200 μl of 0.9% NaCl and injected into the tail vein. Oral applications were via a stomach tube. The instillation solution used was 200 μl 0.9% NaCl, containing 5 μCi He-PC (specific activity: 10 μCi/μmol). After various time intervals, the animals were killed by cervical dislocation. The kidney, lung, liver, spleen, small and large intestine, heart, stomach, thymus, blood, brain and a sample of hind-leg muscle were removed, weighed and combusted in an incinerator (Packard). The percentage radioactivity in the blood was calculated under the assumption that blood represents 7.5% of the total mouse body weight (6). Radioactivity was determined in a Mark III scintillation counter (Searle).

Metabolism. Female NMRI mice (25 g) received 50 μCi of He-PC (specific activity: 105 μCi/μmol) in the tail vein. After 24 or 72 hr, the animals were killed and their livers removed and homogenized in 0.9% NaCl (1:3, w/v). Lipid extraction was done according to Bligh and Dyer (7). The organic phase was evaporated, dried, and the residue redissolved in methanol/chloroform (9:1, v/v). The organic material was applied to a silica gel thin layer chromatography (TLC) plate, and the plate was developed in chloroform/methanol/ammonia (50:50:5, by vol). The upper inorganic phase of the Bligh/Dyer extract also was dried, redissolved in water and applied to a cellulose TLC plate (Merck). The plate was developed in tert butanol/water/acetic acid (90:40:5, by vol). After drying, the plates were scanned for radioactivity using an Automatic Linear Analyzer LB 284 complemented with a Data System LB 500 (Berthold, FRG). For comparison with unlabeled reference compounds, the plates were stained with iodine vapor.

RESULTS

The time-dependent distribution of total radioactivity in different organs after IV injection and oral application is shown in Tables 1 and 2. After IV administration of He-PC, the blood radioactivity showed an initial half-life of ca. 90 min. However, after 192 hr, 12% of the initial radioactivity still remained in the blood indicating a slow elimination of the compound from the blood stream. The highest amounts of radioactivity were recovered from the kidney, liver and lung. Accumulation of the radioactivity in the kidney far exceeded the enrichment of activity in other organs; after 5 hr, the activity in the kidney was 25 times higher than in the blood. In contrast, most other

TABLE 1

Distribution of He-PC in NMRI Mice after IV Administration

	Time						
Organ	10 min	1 hr	5 hr	24 hr	48 hr	96 hr	192 hr
Blood	4.9	2.6	1.8	1.4	1.0	0.7	0.6
Kidney	19.7	31.8	45.7	34.8	17.2	19.2	10.1
Lung	10.9	8.0	6.8	6.1	5.0	3.4	2.6
Liver	10.0	12.6	9.6	8.5	7.9	7.1	5.4
Spleen	5.9	4.0	3.3	3.6	2.6	1.9	1.4
Small intestine	4.4	4.7	5.4	4.5	3.2	2.5	1.7
Heart	3.8	2.3	1.7	1.8	1.4	1.3	1.0
Stomach	3.2	3.0	3.3	4.1	3.2	2.3	1.7
Large intestine	2.8	2.8	3.0	2.7	2.0	1.6	1.2
Thymus	2.2	2.5	2.1	2.7	2.3	1.7	1.7
Muscle	1.6	1.0	0.7	0.8	0.7	0.6	0.6
Brain	0.4	0.3	0.3	0.5	0.5	0.7	1.2

Data represent percent of total radioactivity/g organ (wet weight). S.D. were less than 20%, number of experiments: 3–10 for each time point. For details, see Materials and Methods.

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TABLE 2

Distribution of He-PC in NMRI Mice after Oral Administration

Organ	Time						
	10 min	1 hr	5 hr	24 hr	48 hr	96 hr	192 hr
Blood	0.2	0.4	1.8	1.8	1.5	0.9	0.9
Kidney	0.2	2.0	23.0	34.0	26.2	14.5	11.8
Lung	0.3	0.8	8.1	8.3	6.4	4.1	3.0
Liver	0.3	1.3	10.8	14.7	12.4	10.2	5.8
Spleen	0.1	0.6	4.5	5.0	3.2	2.4	1.8
Small intestine	18.6	17.4	24.5	8.4	4.0	2.8	1.8
Heart	0.1	0.3	1.7	2.6	1.9	1.4	1.3
Stomach	11.4	7.5	4.4	5.0	3.6	2.6	1.9
Large intestine	0.4	0.4	3.3	3.8	2.7	2.0	1.3
Thymus	0.1	0.4	2.3	6.1	3.5	2.8	2.0
Muscle	0.1	0.2	0.7	1.1	1.0	0.8	0.9
Brain	0.02	0.05	0.2	0.6	0.6	0.9	1.3

Data represent percent of total radioactivity/g organ (wet weight). S.D. were less than 20%, number of experiments: 3-10 for each time point. For details, see Materials and Methods.

TABLE 3

Metabolism of Hexadecylphospho(methyl-^{3}H)choline in Mouse Liver after IV Administration

	Time	
	24 hr	72 hr
μCi/g liver	4.0	3.8
Metabolites (%)		
He-PC	63	37
Diacyllecithin	2	7
Phosphocholine	3	3
Choline	32	53

Data represent the means of two experiments for the indicated times.

organs contained similar but lower levels of radioactivity at the different time points measured. In contrast to the other organs, the brain showed a distinct increase in radioactivity up to 192 hr. After oral administration, He-PC behaved similar to the IV route. Again, there was a large enrichment of radioactivity in the kidney, liver and lung.

The metabolic fate of He-PC was studied in detail in the liver. Twenty-four hr after IV injection, the liver contained ca. 63% of the extractable radioactivity as a parent compound; 37% of activity was found to be associated with metabolic breakdown products. As shown in Table 3, the main metabolite of He-PC was choline (32%), whereas phosphocholine (3%) and 1,2-diacylphophatidylcholine (2%) were labeled to a much smaller extent. After 72 hr, the total radioactivity recovered from the liver was decreased slightly. However, the formation of labeled choline and 1,2-diacylphosphatidylcholine increased to 53% and 7%, respectively; phosphocholine remained unchanged.

DISCUSSION

The data presented in this study demonstrate that He-PC irrespective of the administration route is distributed throughout the body. It appears that the compound is efficiently absorbed as such from the intestinal tract and thus makes oral application possible. A calculation of the amount of He-PC absorbed from the gut currently is not possible because, in this experiment, total blood radioactivity was measured. The data given represent the radiolabeled parent compound and radiolabeled metabolites.

Organs showing a large enrichment of He-PC after either route of administration are the liver, lung and kidney. This finding is in line with the biodistribution reported for alkyl lysophospholipids such as 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (OM-GPC) (8). However, in contrast to ALP, the accumulation of He-PC in the kidney is three times higher than that of OM-GPC. This suggests that the kidney may be a target organ for the toxic effects of He-PC in treatment protocols. Comparing the distribution of He-PC in different organs, it is noteworthy that the brain shows a distinct accumulation of radioactivity through the entire period (Tables 1 and 2). We do not know yet if this is due to accumulation of the parent compound or of choline, the latter being one of the main products of He-PC catabolized on the liver. Choline cannot be synthesized in the brain and must be obtained from the blood stream (9).

Metabolism of He-PC in the liver results in the time-dependent formation of radiolabeled choline, phosphocholine and 1,2-diacylphosphatidylcholine. This suggests the importance of the phospholipases C and D in He-PC catabolism. Studies on the in vitro hydrolysis of He-PC with phospholipase C *(Bacillus cereus)* and phospholipase D (partially purified from cabbage) support the finding that APC are substrates for these enzymes (10,11). Formation of 1,2-diacylphosphatidylcholine labeled in the polar head group is consistent with earlier results obtained with OM-GPC. In the latter study, it was shown that the labeled phosphocholine group was transferred from OM-GPC to 1,2-diacylglycerol, resulting in the formation of phosphatidylcholine (3).

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Therapeutic Activity of ET-18-OCH$_3$ and Hexadecylphosphocholine Against Mammary Tumors in BD-VI Rats[1]

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The present therapy experiments with two different transplantable mammary tumors were performed to compare the therapeutic efficacy in BD-VI rats of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH$_3$) and hexadecylphosphocholine (HPC). Both compounds were administered orally, subcutaneously or intracutaneously at equimolar doses ranging from 4.8 to 88 μmol/kg/day five times per week for two weeks. Under the experimental conditions, both transplanted mammary carcinomas were moderately sensitive to the therapy with either HPC or ET-18-OCH$_3$. Comparing both tumors, TMA2 was more sensitive than TMA1. The activity and toxicity of both compounds were dose-related in both tumor lines. Females seemed to be less sensitive with respect to antineoplastic activity and toxicity. Like ET-18-OCH$_3$, HPC was active also at low, probably noncytotoxic doses associated with no detectable toxicity according to body weight development. This suggests that there are at least two different mechanisms of action that lead to tumor growth inhibition.
Lipids 22, 927-929 (1987).

The therapeutic efficacy of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18)OCH$_3$) as a representative of alkyl lysophospholipids was previously tested in several tumor models in vivo and in vitro (1-3). Hexadecylphosphocholine (HPC) belongs to a new class of compounds, the alkylphosphocholines. Originally HPC was synthesized as a "negative control," as this compound was not supposed to be cleaved by the *O*-alkyl cleavage enzyme. Its anticancer activity has so far been tested only against autochthonous tumor models such as the MNU-induced mammary carcinoma, the benzo(a)pyrene-induced fibrosarcoma and the acetoxymethylmethylnitrosamine-induced colorectal carcinoma. The results of these studies are reported by Muschiol et al. (4).

Chemically induced autochthonous mammary carcinomas are a mixture of neoplasms of different morphology and malignancy, especially when they appear at multiple sites. We therefore simplified the system by using transplantable tumors and an inbred animal strain.

The aim of these investigations was to assess the effect of these compounds against homologous mammary tumors. We also wanted to find out to what extent female hormones have an influence on therapy and therefore used rats of both sexes.

MATERIALS AND METHODS

Isogenic female and male BD-VI rats were used as experimental animals. Two invasively growing methylnitrosourea-induced mammary adenocarcinomas, TMA1 and TMA2, were transplanted to female BD-VI rats. The experiments were performed with passages 5-8 of these tumor lines. Tumor cells derived from the donor animals were transplanted subcutaneously (s.c.) or intraperitoneally (i.p.). Therapy started when the tumor diameter averaged 1 cm. The chemical compounds ET-18-OCH$_3$ and HPC were supplied by P. G. Munder (Freiburg, FRG) and H. J. Eibl (Goettingen, FRG), respectively. Equimolar doses of both compounds were administered orally by gavage, intracutaneously (i.c.) or s.c. The animals were treated five times per week for two weeks, then were killed and dissected. As a therapeutic parameter we used T/C%, i.e., the quotient of the tumor-volume difference between treated animals and the control group × 100 (evaluation at week 2).

RESULTS

Table 1 shows the treatment of female BD-VI rats bearing s.c.-transplanted TMA1 or TMA2 tumors by oral administration of equimolar doses of HPC and ET-18-OCH$_3$. The activity and toxicity of both compounds were dose-related in both tumor lines except for treatment with HPC in TMA1 tumor-bearing rats. Moderate antineoplastic activity of high doses was correlated with rather high toxicity. The TMA2 tumor was more sensitive to active dosags of ET-18-OCH$_3$ and HPC than TMA1.

Table 2 shows the treatment of male BD-VI rats bearing i.p.-transplanted TMA1 tumors by oral administration of ET-18-OCH$_3$ and HPC at the same dosages as shown in Table 1. Identical doses caused increased toxicity following i.p. compared to s.c. transplantation. The anticancer efficacy was similar following both dosages of ET-18-OCH$_3$, whereas a dose-response relationship was observed following treatment with HPC.

Tables 3 and 4 show several experiments in which males and females bearing s.c.-transplanted TMA1 or TMA2 tumors were treated with equimolar doses of ET-18-OCH$_3$ or HPC. On a mg/kg body weight basis, females seemed less sensitive than their male counterparts with respect to anticancer activity and toxicity observed after treatment with HPC or ET-18-OCH$_3$. The experiments again demonstrated that the TMA2 tumor was more sensitive to therapy than the TMA1 tumor. The moderate antineoplastic activity and toxicity seen in treated male animals and assessed by body weight development was dose-related for both tumor lines.

Table 5 illustrates the treatment of male BD-VI rats bearing s.c.-transplanted TMA1 or TMA2 tumors by i.c. administration of HPC. Therapy with low doses of HPC was associated with significant anticancer efficacy on both TMA1 and TMA2 s.c.-growing tumors, while lower toxicity was observed.

DISCUSSION

Under the experimental conditions used, the two transplanted mammary carcinomas TMA1 and TMA2 were moderately sensitive to the therapy with HPC or ET-18-OCH$_3$. The different administration modes are

[1]Presented at the symposium on "Ether Lipids in Oncology," Göttingen, Federal Republic of Germany, December 1986.

TABLE 1

Treatment of Female BD-VI Rats Bearing Subcutaneously (s.c.) Transplanted TMA1 or TMA2 Tumors by Oral Administration of HPC or ET-18-OCH_3

Tumor line s.c.	No. of animals/group	Dose schedule (mg/kg/day)	T/C% at evaluation	Body wt difference (%)	Mortality[a] (%)
TMA1	13	45 ET-18-OCH_3	46	−7	0
TMA1	13	22.5 ET-18-OCH_3	66	+1	15
TMA1	13	36 HPC	89[b]	−10	46
TMA1	13	18 HPC	66	−4	15
TMA1	13	Control	100	+5	0
TMA2	10	45 ET-18-OCH_3	51	−8	30
TMA2	10	22.5 ET-18-OCH_3	92[b]	−1	0
TMA2	10	36 HPC	32	−15	0
TMA2	10	18 HPC	65	−8	0
TMA2	10	Control	100	+3	0

[a]Due to drug toxicity.
[b]Not significant according to Student's t-test.

TABLE 2

Treatment of Male BD-VI Rats Bearing Intraperitoneally (i.p.) Transplanted TMA1 Tumors by Oral Administration of HPC or ET-18-OCH_3

Tumor line i.p.	No. of animals/group	Dose schedule (mg/kg/day)	T/C% at evaluation	Body wt difference (%)	Mortality[a] (%)
TMA1	16	45 ET-18-OCH_3	64	−24	56
TMA1	16	22.5 ET-18-OCH_3	63	−13	25
TMA1	16	36 HPC	52	−15	19
TMA1	16	18 HPC	104[b]	−11	13
TMA1	16	Control	100	+1	0

[a]Due to drug toxicity.
[b]Not significant according to Student's t-test.

TABLE 3

Treatment of Male BD-VI Rats Bearing Subcutaneously (s.c.) Transplanted TMA1 or TMA2 Tumors by s.c. Administration of HPC or ET-18-OCH_3

Tumor line s.c.	No. of animals/group	Dose schedule (mg/kg/day)	T/C% at evaluation	Body wt difference[a] (%)
TMA1	10	20 ET-18-OCH_3	42	−8
TMA1	10	10 ET-18-OCH_3	81[b]	−3
TMA1	10	15.6 HPC	48	−8
TMA1	10	7.8 HPC	64	−3
TMA1	10	Control	100	+1
TMA2	10	20 ET-18-OCH_3	24	−11
TMA2	10	10 ET-18-OCH_3	47	−4
TMA2	10	15.6 HPC	46	−10
TMA2	10	7.8 HPC	73[b]	−6
TMA2	10	Control	100	+3

[a]No drug-related mortality.
[b]Not significant according to Student's t-test.

TABLE 4

Treatment of Female BD-VI Rats Bearing Subcutaneously (s.c.) Transplanted TMA1 or TMA2 Tumors by s.c. Administration of HPC or ET-18-OCH_3

Tumor line s.c.	No. of animals/group	Dose schedule (mg/kg/day)	T/C% at evaluation	Body wt difference[a] (%)
TMA1	6	20 ET-18-OCH_3	87[b]	0
TMA1	6	10 ET-18-OCH_3	71	+1
TMA1	6	15.6 HPC	92[b]	−3
TMA1	6	7.8 HPC	68	−9
TMA1	6	Control	100	+2
TMA2	6	20 ET-18-OCH_3	74[b]	−2
TMA2	6	10 ET-18-OCH_3	73[b]	−4
TMA2	6	15.6 HPC	54	−4
TMA2	6	7.8 HPC	159[c]	−2
TMA2	6	Control	100	−4

[a]No drug-related mortality.
[b]Not significant according to Student's t-test.
[c]Significant increase according to Student's t-test.

TABLE 5

Treatment of Male BD-VI Rats Bearing Subcutaneously (s.c.) Transplanted TMA1 or TMA2 Tumors by i.c. Administration of HPC

Tumor line s.c.	No. of animals/group	Dose schedule (mg/kg/day)	T/C% at evaluation	Body wt difference[a] (%)
TMA1	10	7.8 HPC	51	−7
TMA1	10	3.9 HPC	78	−5
TMA1	10	1.95 HPC	76	−4
TMA1	10	Control	100	−4
TMA2	10	7.8 HPC	66	−2
TMA2	10	3.9 HPC	58	0
TMA2	10	1.95 HPC	64	+2
TMA2	10	Control	100	+4

[a]No drug-related mortality.

comparable because high resorption was found for HPC (5). Comparing the tumor lines, TMA2 was more sensitive than TMA1. Female animals seemed less sensitive than males with respect to antineoplastic activity as well as toxicity. ET-18-OCH_3 is known to be active at high cytotoxic concentrations, probably due to interaction with cellular membranes, and at low, probably non-cytotoxic, doses, which seem to influence the immune response (1–3). Although we did not measure the immune response following treatment with ET-18-OCH_3 and HPC in this experiment, we suppose that the treatment-related effects of both compounds follow similar modes of action at low dosages.

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Alkyl Phosphocholines: Toxicity and Anticancer Properties[1]

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The study reports on the investigation of acute and subacute toxicity and on antineoplastic activity of hexadecylphosphocholine (HPC), the first compound of a new class of antineoplastic chemotherapeutics. In rats, the LD_{50} of HPC was 606 μmol/kg; the maximum tolerable dose over four weeks was 39 μmol/kg. Symptoms of toxicity were enteritis, spider cell activation in the liver, hemosiderosis in the spleen and reversible transaminase increase. The best therapeutic effect was observed on methylnitrosourea (MNU)-induced mammary carcinoma in the rat. Two transplantable mammary carcinomas in the rat and autochthonous benzo(a)pyrene-induced sarcomas exhibited low-grade sensitivity to HPC. The MXT mammary carcinoma of the mouse, the Walker 256 carcinosarcoma of the rat, and autochthonous acetoxymethylmethylnitrosamine-induced colonic tumors of the rat were not chemosensitive to HPC.
Lipids 22, 930-934 (1987).

In the course of assessing the toxicological and preclinical therapeutic value of alkyl lysophospholipids, we also investigated hexadecylphosphocholine (HPC). The experimental results of the toxicity and therapy studies with HPC are described below.

MATERIALS AND METHODS

Chemicals. Methylnitrosourea (MNU) and acetoxymethylmethylnitrosamine (AMMN) were synthesized by M. Wiessler (Institute of Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg, FRG). Benzo(a)pyrene (BaP) was purchased from Sigma (Munich, FRG). The alkylphosphocholine HPC was synthesized by H. J. Eibl (Max Planck Institute of Biophysical Chemistry, Göttingen, FRG). All compounds were dissolved shortly before use at adequate concentrations.

Animals and tumor induction. Male and female Sprague-Dawley rats (Zentralinstitut für Versuchstierkunde, Hannover, FRG) were kept under conventional, controlled conditions. Autochthonous mammary carcinomas (1), colorectal carcinomas (2-4) and fibrosarcomas (5) were induced as described previously. TMA1 and 2 mammary carcinomas were transplanted in BD VI rats as described elsewhere in this issue (6). MXT mammary carcinoma was processed for treatment by s.c. implanting 30 mg tumor tissue into female $B_6D_2F_1$ mice. Treatment started at defined stages of macroscopically visible tumor development. Comparison of drug effectiveness was based on the increase in median survival time (ILS) and on differences in tumor volumes expressed as percent of untreated control (T/C%). National Cancer Institute criteria were used for assessing the activity of the compounds evaluated (7). Statistical evaluation of tumor growth was performed using a non-parametric multivariate test (8) and Student's t-test.

Evaluation of toxicity. For acute toxicity studies, HPC was administered p.o. to Sprague-Dawley (SD) rats that had been fasted overnight. Surviving animals were observed for 14 days. LD_{50} values were determined by the method of Spearman and Kärber (9). Additionally, subacute toxicity (four weeks) was determined in SD rats and in dose-finding experiments in white New Zealand rabbits and emetic effects in beagle dogs.

RESULTS

Toxicological studies. In experiments in rats, the acute LD_{50} after oral administration of HPC by gavage to four groups of 10 adult Sprague-Dawley rats of either sex was 246 mg/kg body weight (bw) (corresponding to 606 μmol/kg) (Table 1). The symptoms of intoxication were sedation, salivation, weight loss and diarrhea. Dissection of those in the high-risk group revealed intestinal rubor, swelling of the intestinal mucosa and ulcers of the

TABLE 1

Summary of Toxicity Experiments

	Species	Strain	Sex
Acute toxicity[a]	Rat	Sprague-Dawley	Female/male
Subacute toxicity[b]	Rat	Sprague-Dawley	Female/male
Subchronic toxicity	Rat	Sprague-Dawley	Female/male
Determination of tolerable dose	Rabbit	White New Zealand	Female
Emetic effect	Dog	Beagle	Female

[a]LD_{50} = 606 μmol/kg (246 mg/kg) according to Spearman and Kärber (7).
[b]Maximum-tolerated dose = 39 μmol/kg (16 mg/kg).

TABLE 2

Subacute Toxicity

Dose μmol/kg	Sex	No. of animals	Mortality %	%BWD[a]
0	Female	20	0	−2.6
0	Male	20	0	+1.6
62	Female	20	10	−5.2
62	Male	20	20	−13.9
49	Female	20	5	−10.7
49	Male	20	5	−4.5
39	Female	20	0	−3.4
39	Male	20	0	−1.1

[a]Median body weight difference (week 4-week 1) in % of initial weight.

[1]Presented at the symposium on "Ether Lipids in Oncology," Göttingen, Federal Republic of Germany, December 1986.
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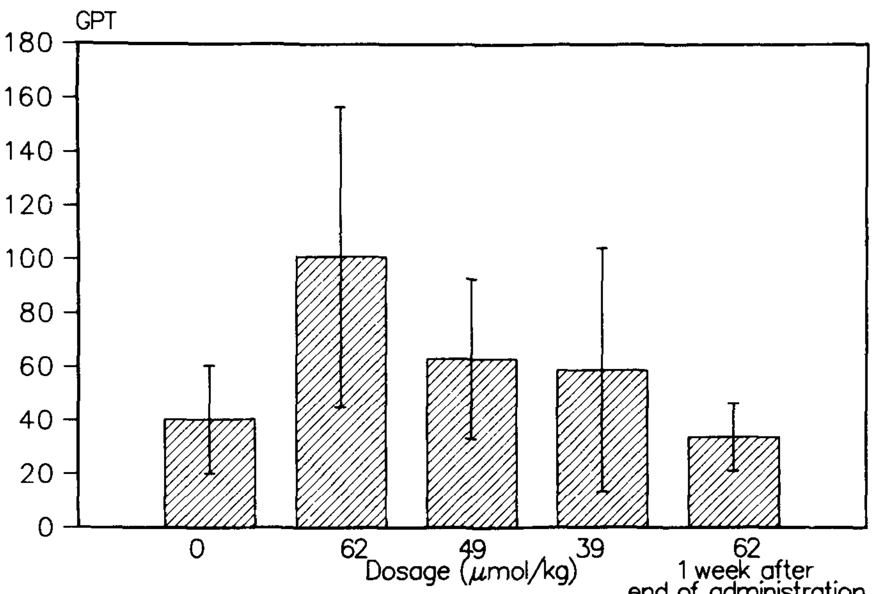

FIG. 1. Level of glutamic acid pyruvic transaminase (GPT) after administration of HPC (subacute toxicity).

forestomach. Animals generally died two to three days after the single dose of HPC. Histopathological examination confirmed the enterocolitis diagnosed macroscopically. No additional symptoms were seen. In the final analysis, death occurred from desiccation and possible toxin absorption.

In another experimental design, the subacute toxicity of HPC was determined in a four-week experiment (Table 2). The four experimental groups each consisted of 20 male and 20 female adult SD rats. The daily doses administered amounted to 0, 16, 20 and 25 mg/kg bw, respectively. No deaths occurred in the lowest dose group (16 mg/kg) during the observation. Unlike this, significant retardation in weight development was seen in the two high-dose groups. Five percent of the animals in the 20 mg/kg dose group died, and 10% of the females and 20% of the males in the highest-dose group died. The symptoms of intoxication corresponded to those observed in the acute toxicity experiment. As expected, the intoxication was clearly dose-related. The histopathological examinations disclosed activated spider cells in the liver and hemosiderosis and enterocolitis in the spleen. Damage to the kidneys, such as nephrosis, pyelonephritis and epithelial hyperplasia, were also found in animals of all treated groups. These changes were seen in about one-third of the animals. According to the findings, the lowest dose is considered the maximum-tolerable dose (MTD).

In accordance with clinical chemical investigations in the acute and subacute toxicity experiments, transaminases increased two- to threefold the normal value (Fig. 1) but normalized when the administration was discontinued. The other parameters determined by clinicochemical examination (differential hemogram, erythrocytes, leukocytes, hematocrit, MCV, Hb, LDH, kreatinine, Na, K, Ca) did not deviate from normal.

Additionally, a subchronic three-month experiment, in which doses of 6, 10, 16 and 20 mg/kg bw were administered, was carried out. The findings have not been

TABLE 3

Emetic Effect of Hexadecylphosphocholine (HPC) in Beagle Dogs

Dog no.	Gelatinous capsules	Dose μmol/kg	Emetic effect
1	Soluble in the stomach	12.3[a]	−
1	Soluble in the stomach	24.5[b]	+
2	Soluble in the stomach	12.3	+
2	Soluble in the stomach	24.5	+
1	Soluble in the small intestine	24.5	−
2	Soluble in the small intestine	24.5	−

[a]Equivalent to 5 mg/kg.
[b]Equivalent to 10 mg/kg.

evaluated completely yet but the trend is that the MTD is about 10 mg HPC/kg bw in males and females.

Rabbits were used only for a dose-finding test on acute toxicity (Table 1). Two adult female rabbits tolerated a dose of 100 mg/kg bw five times a week without any symptoms of diarrhea. Thus, the response in rabbits was not more sensitive than in rats.

Two female beagle dogs were used for testing only emetic effects of HPC (Table 3). Single doses of 5 and 10 mg/kg bw were administered by means of capsules that were soluble in the stomach or the small intestine. Emesis resulted only after administering the high dose of capsules soluble in the stomach but not after capsules soluble in the small intestine. Consequently, possible emetic effects might be avoided by inhibiting the release of HPC in the stomach.

Therapeutic studies. These were carried out, using several experimental tumor models (Tables 4-7). The autochthonous MNU-induced mammary carcinoma in Sprague-Dawley rats was investigated in detail (1,8) (Table 4). This adenocarcinoma again proved to be a

TABLE 4

Treatment of Methylnitrosourea-induced Rat Mammary Carcinoma with Hexadecylphosphocholine (HPC)

	No. of animals	Dose	T/C%[a]	%ILS[b]	%BWD[c]	%Mortality
Control 1	20	0	100	0	+11	5
HPC[d]	15	115	toxic	−69	toxic	93
	15	77	2.3	+26	−4	40
	15	51	13.2	+23	+2	0
	15	34	38.6	+38	+8	7
	15	15	27.7	+24	+8	10
	15	7	74.0	+16	+2	10
Control 2	20	0	100	0	+6	15
HPC[e]	15	115	11.6	+66	−3	14

[a]T/C% = quotient of median tumor volume of treated and control group × 100 (evaluation week 6).
[b]ILS% = % increase in median life span over control.
[c]BWD% = median body weight difference (week 6–week 1) in percent of initial weight.
[d]HPC = continuous oral treatment (5×/week × 5 weeks).
[e]HPC = intermittent oral treatment (5×/week, every second week).

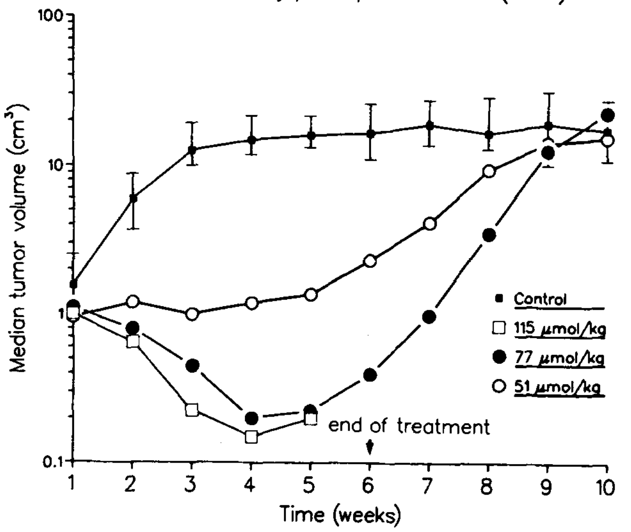

FIG. 2. Treatment of MNU-induced mammary carcinoma with hexadecylphosphocholine (HPC).

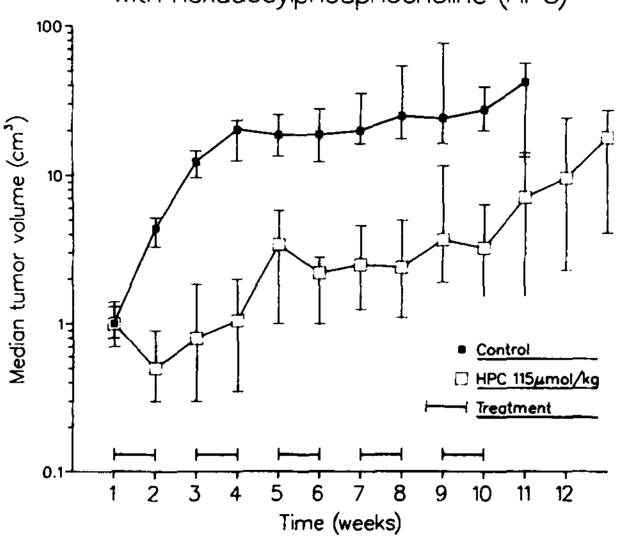

FIG. 3. Intermittent treatment of MNU-induced mammary carcinoma with hexadecylphosphocholine (HPC).

suitable model for testing the therapeutic efficacy of drugs because of its surprisingly sensitive response to HPC. Fifty-one µmol/kg HPC, corresponding to 21 mg/kg bw, was the optimum dose. It yielded complete remissions lasting over the whole administration period, but it did not induce toxic reactions. The tumor volume increased again when the administration was discontinued (Fig. 2). After renewed administration of HPC, the tumor growth again came to a standstill. This indicates that no resistance was induced (Fig. 3).

The experiments on transplantable mammary carcinomas of the rat (Table 5) revealed moderate sensitivity of the TMA 1 and 2 tumors, whereas the Walker 256 carcinosarcoma did not respond to the HPC therapy. The MXT mammary carcinoma of the mouse was similarly insensitive (Table 6).

The growth of another autochthonous tumor, i.e. the colonic carcinoma of the rat (2–4), was not inhibited by HPC despite administration of maximum doses (Table 7).

Yet another autochthonous tumor was investigated: the benzo(a)pyrene-induced sarcoma of the rat (5). The results are presented in Figure 4. Comparatively good chemosensitivity of the tumors was observed in some animals; others showed no response. Therefore, mere comparison of the mean tumor growth curves would reveal little difference to the untreated control during the period of treatment. However, evaluation by Koziol and Donna of the growth development using the multivariate test proved

TABLE 5

Efficacy of Hexadecylphosphocholine (HPC) Against Transplantable Mammary Tumors

Tumor	Route of implantation	No. of animals	Sex	Application route	Dose µmol/kg[a]	T/C%[b]	Significance[c]
TMA1	sc	10	Male	sc	38.3	48	+
TMA1	sc	10	Male	sc	19.1	64	+
TMA1	sc	6	Female	sc	38.3	92	–
TMA1	sc	6	Female	sc	19.1	68	+
TMA1	sc	10	Male	ic	19.1	51	+
TMA1	sc	10	Male	ic	9.6	78	+
TMA1	sc	10	Male	ic	4.8	76	+
TMA1	sc	13	Female	po	88	89	–
TMA1	sc	13	Female	po	44.2	66	+
TMA1	ip	16	Male	po	88	52	+
TMA1	ip	16	Male	po	44.2	104	–
TMA2	sc	10	Male	sc	38.3	46	+
TMA2	sc	10	Male	sc	19.1	73	(–)
TMA2	sc	6	Female	sc	38.3	54	+
TMA2	sc	6	Female	sc	19.1	159	–
TMA2	sc	10	Male	ic	19.1	66	+
TMA2	sc	10	Male	ic	9.6	58	+
TMA2	sc	10	Male	ic	4.8	64	+
TMA2	sc	10	Female	po	88	32	+
TMA2	sc	10	Female	po	44.2	65	+
Walker	sc	10	Female	po	115	108	–
Walker	sc	10	Female	po	77	108	–
Walker	sc	10	Female	po	51	93	–

[a]5×/week × 2 weeks.
[b]T/C% = Quotient of tumor/volume-difference of treated and control group × 100 (evaluation week 2).
[c]+ = significant according to Student's t-test.

TABLE 6

Treatment of MXT Mammary Carcinoma[a] with Hexadecylphosphocholine (HPC)

Treatment	No. of animals	Dose[b] µmol/kg	T/C% week 3	Mortality% week 3
HPC	15	147	78	40
HPC	15	74	91	13
Control	17	/	/	6

[a]sc implantation of 30 mg tumor tissue into female $B_6D_2F_1$ mice.
[b]Treatment po 5×/week × 3 weeks.

TABLE 7

Treatment of Acetoxymethylmethylnitrosamine-Induced Colorectal Rat Carcinoma with Hexadecylphosphocholine (HPC)

Treatment	No. of animals	Median tumor volume (95% conf. limits)	Median tumor number (95% conf. limits)	%BWD[a]	%Mortality
Control 1[b]	20	36.5 (24–74)	2 (1–3)	/	/
Control 2[c]	20	389 (264–1046)	4 (3–7)	+7	5
HPC[d]	26	535 (339)–935)[e]	5 (3–7)	−15	54

[a]Median body weight difference (week 11–week 1) in percent of initial weight.
[b]Control terminated at endoscopic diagnosis of tumors.
[c]Control terminated at end of therapy.
[d]98 µmol/kg × 5/week × 10 weeks.
[e]T/C × 100 = 138.

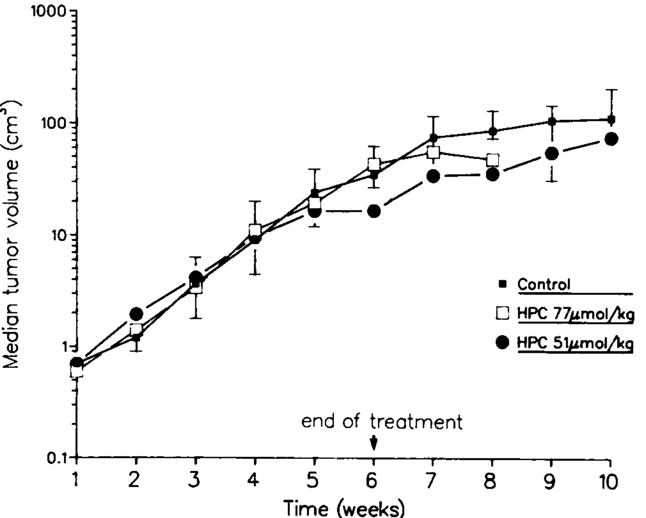

FIG. 4. Treatment of benzo(a)pyrene-induced fibrosarcoma with hexadecylphosphocholine (HPC).

significant tumor-inhibiting effects of HPC at a dose of 51 μmol/kg.

DISCUSSION

The most important finding of the experiments described was that hexadecylphosphocholine affected growth retardation of autochthonous mammary carcinomas in rats. This effect was superior to the inhibitory effect induced, for instance, by cyclophosphamide (1). HPC thus proved to be an interesting new model compound in the development of active anticancer drugs. This is more important because its toxicity is obviously quite different from that of known antineoplastic agents such as alkylating compounds or antimetabolites. Remarkable, though not exceptional, is the fact that the therapeutic activity of HPC manifested itself markedly against only one tumor, i.e. the autochthonous mammary carcinoma of the rat, while other experimental tumors were not sensitive at all. Perhaps this offers the possibility of developing compounds of the described type especially for use against certain organ tumors. Such an "aimed" therapy surely would be more favorable than the "shotgun" therapy that many presently available compounds exert. First clinical results tend to support this hypothesis.

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Modulation of Chemical Carcinogenesis in Rats by Alkyl Lysophospholipids[1]

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The influence of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) and 1-hexadecylmercapto-2-methoxymethyl-*rac*-propyl-3-phosphocholine (TLP, BM41.440) on methylnitrosourea (MNU)-induced rat mammary carcinomas and of ET-18-OCH_3 on 7,12-dimethylbenzanthracene (DMBA)-induced leukemias was investigated. Both agents effectively delayed MNU-induced mammary tumor formation at high, cytotoxic dosages but TLP had no influence at low "immunomodulatory" doses. ET-18-OCH_3 also significantly protected against leukemia development in DMBA-treated Long-Evans rats.
Lipids 22, 935–942 (1987).

Modulation of tumor occurrence by selected drugs is a modern field of research with growing importance. From the two types of effective modulation that either enhance or reduce tumor incidence, the latter type has been investigated by groups of researchers, including ourselves, who are interested in "chemoprevention" of cancer.

Some agents chemically inhibit the reaction of certain carcinogens with desoxyribonucleic acid (DNA) by scavenging or more specific mechanisms of action (1,2). Other compounds are able to interfere later on during the interval between initiation and manifestation of tumors.

Therefore, we investigated the ability of retinoids (3–5) and sulphur-containing agents such as disulfiram (6,7) and mesna (8) to inhibit the formation of certain chemically induced tumors in animals. Reduced fibrosarcoma expression in methylcholanthrene-induced mice following administration of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) (9) prompted us to investigate the potential of alkyl lysophospholipids to modulate chemical carcinogenesis on a larger scale. Two compounds that had shown promising chemotherapeutic properties in animal systems and in first trials in man (10,11) were selected, i.e. ET-18-OCH_3 and 1-hexadecylmercapto-2-methoxymethyl-*rac*-propyl-3-phosphocholine (TLP, BM 41.440, Ilmofosine), as indicated in Figure 1.

This article summarizes our experiments on these two agents with regard to their influence on the occurrence of rat mammary carcinomas induced by N-methylnitrosourea (MNU; experiments A,C) and on the manifestation of rat erythroleukemia induced by 7,12-dimethylbenzanthracene (DMBA; experiment B).

MATERIALS AND METHODS

Chemicals. MNU was synthesized by Dr. M. Wiessler (Institute of Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg) and dissolved at 1% in Sörensen buffer, pH 6 and distilled water (20:80, v/v). DMBA was obtained as a 0.5% fat emulsion (5 mg/ml) (intralipid) from Upjohn Company (Kalamazoo, MI). ET-18-OCH_3 (Fig. 1) was supplied by Paul Munder (Max-Planck Institute for Immunobiology, Freiburg) and dissolved at 0.1% in physiological saline or at 1% in human serum albumin (Human Albumin 20%, Immuno GmbH, Heidelberg) and physiological saline (5.95, v/v). TLP (Fig. 1) was obtained from Boehringer Mannheim (Mannheim, FRG) and dissolved at 0.2% and 3% in tap water. Etretinate (Tigason®) was supplied by Hoffman-LaRoche, Basel and administered as 0.05% solution in tap water contained in light-protected bottles. All solutions were prepared shortly before use.

Animals and tumor induction. Rats were purchased from commercial sources (Zentralinstitut für Versuchstierzucht [Hannover, FRG]) and kept under conventional conditions. Altromin pellets and tap water were given ad libitum.

Induction of mammary carcinomas in SD-rats and of leukemias in LE-rats were performed basically as described elsewhere (12–14). Female SD-rats received a single IV injection of 50 or 25 mg/kg MNU on day 50 of life. Male and female LE-rats were injected with 30, 10, 20 and 10 mg/kg DMBA on days 27, 42, 57 and 70 of life, respectively.

All animals were observed while alive; only moribund animals were sacrificed. Dead animals were dissected. Specimens of macroscopically changed organs were examined histologically to determine tumor incidences and causes of death.

The number of tumors per animal and the induction time for the first tumor of each animal were analyzed by the Krustal-Wallis test to find significant differences (15). Tumor rates were analyzed by comparison of organ-specific, age-adjusted observed vs. numbers of affected animals expected according to Peto et al. (16). The administration schedule of experiments A, B and C are given in Tables 1 and 3 and in Figure 5.

RESULTS

Influence of ET-18-OCH_3 on MNU-induced rat mammary carcinoma. The results of oral administration of ET-18-OCH_3 to female SD-rats induced with MNU are given in Figure 2 and Table 2. Rats receiving 50 mg/kg MNU developed only multiple mammary carcinomas (100%) within 10 weeks after injection of the carcinogen. Two–nine tumors (median: 5) were found per rat at the experiment's termination. The total tumor load per rat was 1.6–45.4 cm^3 (median: 15.9 cm^3). Administration of ET-18-OCH_3 inhibited the occurrence and growth of mammary lesions. After 10 weeks of administration, only 80% of the animals had developed tumors. The size of these tumors was distinctly smaller, ranging from 1–16.2 cm^3 (median: 6.8 cm^3). Moreover, the number of tumors per

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Abbreviations: ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; TLP, 1-hexadecylmercapto-2-methoxymethyl-*rac*-propyl-3-phosphocholine; DMBA, 7,12-dimethylbenzanthracene.

$H_2C-O-(CH_2)_{17}-CH_3$
$H-C-O-CH_3$
$H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

ET-18-OCH₃

$H_2C-S-(CH_2)_{15}-CH_3$
$H-C-CH_2-O-CH_3$
$H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

BM 41.440
TLP

FIG. 1. Chemical structures of 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine ($ET\text{-}18\text{-}OCH_3$) and of 1-hexadecylmercapto-2-methoxymethyl-*rac*-propyl-3-phosphocholine (TLP, BM 41.440, Ilmofosine).

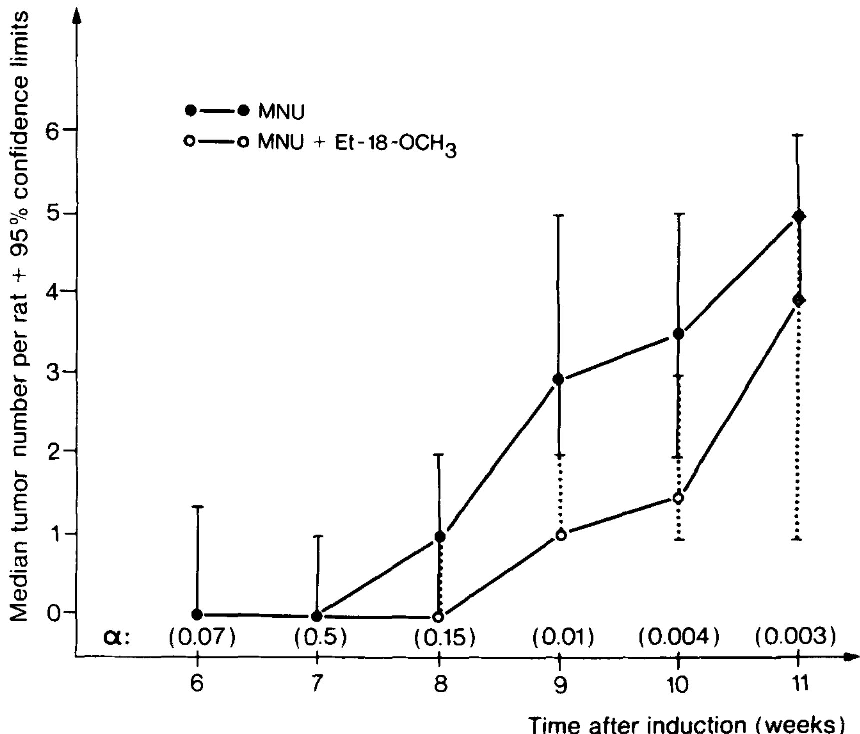

FIG. 2. Increase in median tumor number of MNU-induced rats and protective influence of $ET\text{-}18\text{-}OCH_3$; α corresponds to the standard significance comparing tumor numbers of treated and control animals according to a rank sum test described by Dunn (15).

TABLE 1

Influence of the Alkyl Lysophospholipid $ET\text{-}18\text{-}OCH_3$ on the Carcinogenesis of Female SD-Rats after Induction with Methylnitrosourea

Group no.	Animal no.	Induction with MNU	Treatment	Treatment schedule	Single (total) dose
I	20	50 mg/kg IV	—	—	—
II	20	50 mg/kg IV	$ET\text{-}18\text{-}OCH_3$ po	2× daily day 1-5/week × 10 weeks	10 (1000) mg/kg

tumor-bearing rat was significantly lower during the last three weeks of the experiment (Fig. 2). However, no significant difference was found in the occurrence of the first tumor per rat, the median manifestation time being 43 days in ET-18-OCH_3-treated animals and 44 days in untreated control animals.

Protection by ET-18-OCH_3 against leukemia development in DMBA-treated Long-Evans rats. Induction of LE-rats with DMBA is a highly effective method of generating predominantly erythroleukemias within a short period of time (Fig. 3). These leukemias affect a median survival time of 10 days following diagnosis (range: 2–25 days; Fig. 4). In both experiments (I and II, Table 4), treatment with four pulse doses of DMBA resulted in rapid and almost complete leukemia induction. Median survival times from the start of experiment amounted to 102 days at an incidence rate of 90% in experiment I and 113 days at 96% in experiment II. Regular administration of ET-18-OCH_3 as shown in Table 3, effected an increase in median survival times of 17–53% compared with the respective untreated control (groups Ia–d, Table 4). Later onset of administration appeared to be at least as successful in increasing the median life span as an earlier start of treatment. However, treatment-related reduction in leukemia incidence was observed concomitantly with increased crude incidence of neoplasias of the mammary

TABLE 2

Influence of the Alkyl Lysophospholipid ET-18-OCH_3 on the Carcinogenesis of Female SD-Rats after Induction with Methylnitrosourea

Group no.	Median tumor volume per rat at death [cm^3] (range)	Treated (T) / Control (C) × 100	Median tumor number per rat at death (range)	Median manifestation time [days][c] of the first tumor per rat (range)	Overall incidence N (%)
I	15.9 (1.6–46.4)	100	5 (2–9)	44 (38–65)	20 (100)
II	6.8[a] (1.0–16.2)	42.8	4[b] (1–6)	43 (38–65)	16 (80)

[a]Significant vs I, = 0.01 according to the Kruskal Wallis Test (15).
[b]Significant vs I, = 0.003 according to the Kruskal Wallis Test (15).
[c]Day of induction = day 0.

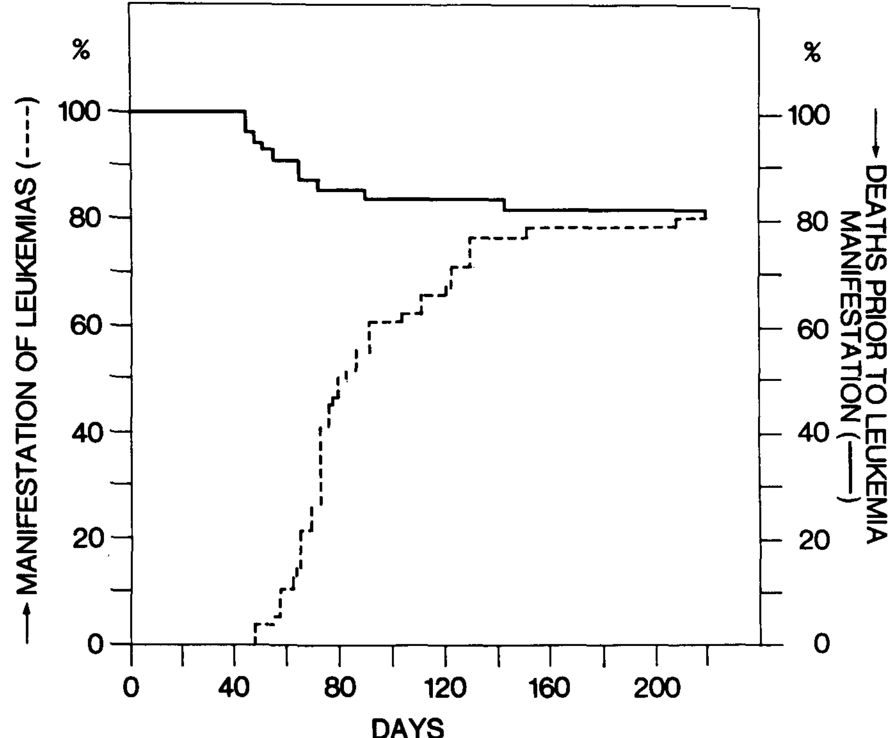

FIG. 3. Incidence of leukemias and toxic deaths in Long-Evans rats following induction with DMBA.

gland (41% vs 16% in untreated controls) and of other malignant tumors (28% squamous cell carcinomas of the ear duct vs 11% in untreated controls). This increase was at least in part due to the longer survival of treated rats. Statistical analysis allowing for differences in survival revealed no significant increase in mammary neoplasias or other tumors. Combined administration of ET-18-OCH_3 and the retinoid etretinate, also a chemopreventive agent (IIb, Table 4) failed to prolong remarkably the lifespan of animals but significantly lowered the incidence of mammary neoplasias.

Suppression of MNU-induced mammary carcinoma by TLP. Since the onset of alkyl lysophospholipid administration influenced the leukemia incidence following DMBA induction, the time schedule of TLP administration was varied (Fig. 5). The period of administration was restricted to a total of 12 weeks. Additionally, the doses of the carcinogen and of TLP were varied to compose both low "immunomodulatory" or high cytocidal doses of TLP and varied to compose tumor induction schemes affecting rapid or delayed tumor manifestation.

The tumor occurrence following MNU alone is shown in Figure 6. At week 40, the tumor incidences were 98% after 50 mg/kg and 85% after 25 mg/kg MNU. The effect of the low TLP dose is given in Figure 7. The manifestation of mammary lesions induced with 50 mg/kg MNU clearly was not suppressed by a 12-week administration of TLP. Tumor occurrence was slightly delayed

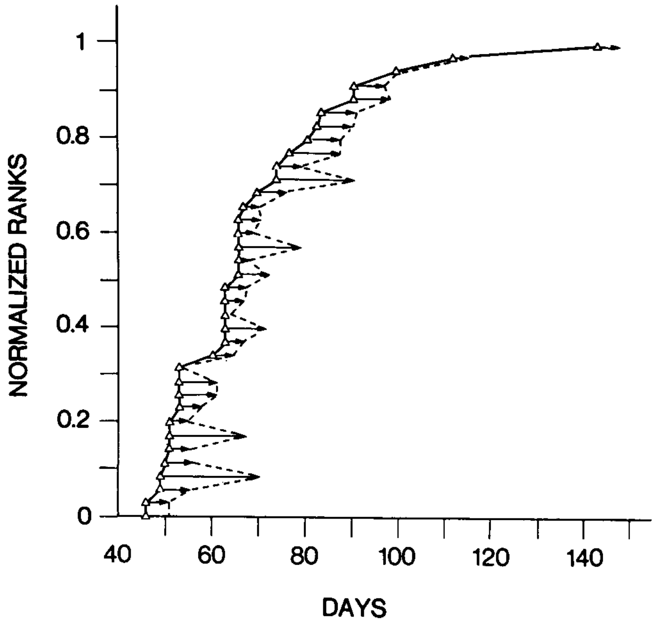

FIG. 4. Survival time of leukemic LE-rats from diagnosis (open triangles) until death (tip of arrow).

TABLE 3

Design of the Experiment Investigating the Influence of the Alkyl Lysophospholipid ET-18-OCH_3 on 7,12-Dimethylbenzanthracene-Induced Leukemogenesis in LE-Rats

Group no.	No. of animals	Treatment[a]	Daily single dose (mg/kg)
I a	100	Untreated control	—
b	20	ET-18-OCH_3 from day 28	20
c	29	ET-18-OCH_3 from day 43	20
d	29	ET-18-OCH_3 from day 58	20
e	30	ET-18-OCH_3 from day 71	20
II a	48	Untreated control	—
b	32	ET-18-OCH_3 from day 28	20
		+	
		Etretinate from day 28	5

[a]All animals received injections of DMBA on day 27, 42, 57 and 70 of life for induction of leukemia.

TABLE 4

Influence of the Alkyl Lysophospholipid ET-18-OCH_3 on 7,12-Dimethylbenzanthracene-Induced Leukemogenesis in LE-Rats

			Tumor occurrence					
Group no.	Median survival time (days)[a]	% ILS[b]	Leukemias N (%)	p[c]	Mammary tumors N (%)	p[c]	Others[d] N (%)	p[c]
I a	102	—	90 (90)	—	16 (16)	—	11 (11)	—
b	119	+ 17	17 (85)	0.08	5 (25)	0.73	3 (15)	0.3
c	120	+ 18	26 (90)	0.11	8 (28)	0.19	2 (7)	0.07
d	156	+ 53	22 (76)	0.0001	12 (41)	0.08	8 (28)	0.09
e	125	+ 23	26 (87)	0.18	7 (23)	0.32	1 (3)	0.053
II a	113	—	46 (96)	—	11 (23)	—	1 (2)	—
b	120	+ 6	27 (84)	0.13	2 (6)	0.04	1 (3)	0.86

[a]From start of experiment = day 27 of life.

[b]% Increase in median life span over untreated controls.

[c]2-Tailed p-value comparing treated with control animals, using the method described by Peto et al. (16) for tumors discovered in a fatal context.

[d]Mainly squamous cell carcinomas of the external auditory canal.

in rats by application starting at day 21 after induction. However, the higher dose of TLP suppressed the tumor growth for a considerable time (Fig. 8). The growth inhibition was maintained for three to seven weeks after the end of the respective treatment period.

Longer delay in tumor manifestation was seen when the higher dose of TLP was administered to rats who were induced with 25 mg/kg MNU (Fig. 9). In comparison with the higher induction scheme, the observed suppression was relatively stronger. At week 40, a 68% reduction in

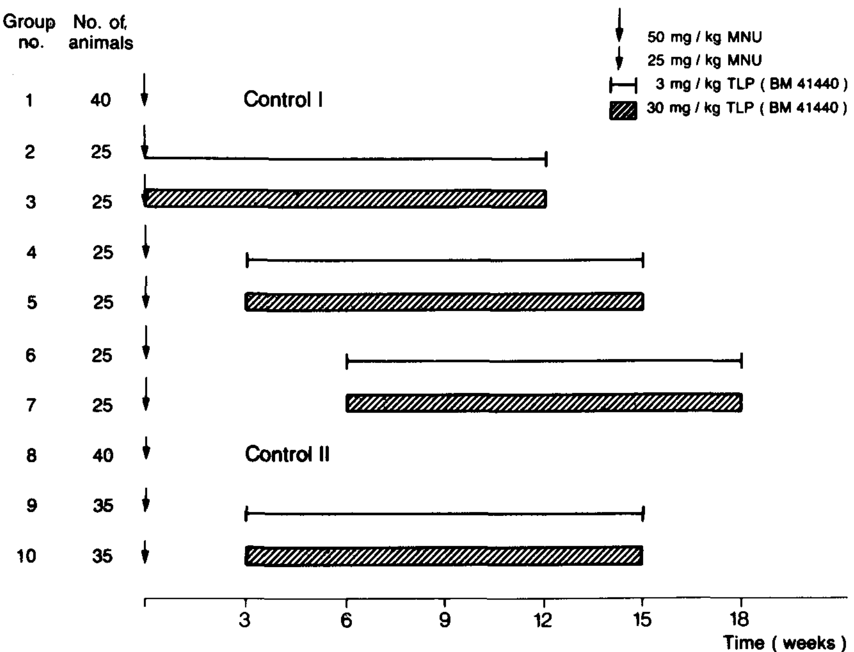

FIG. 5. Experimental design of administering TLP to groups of female SD-rats induced with MNU.

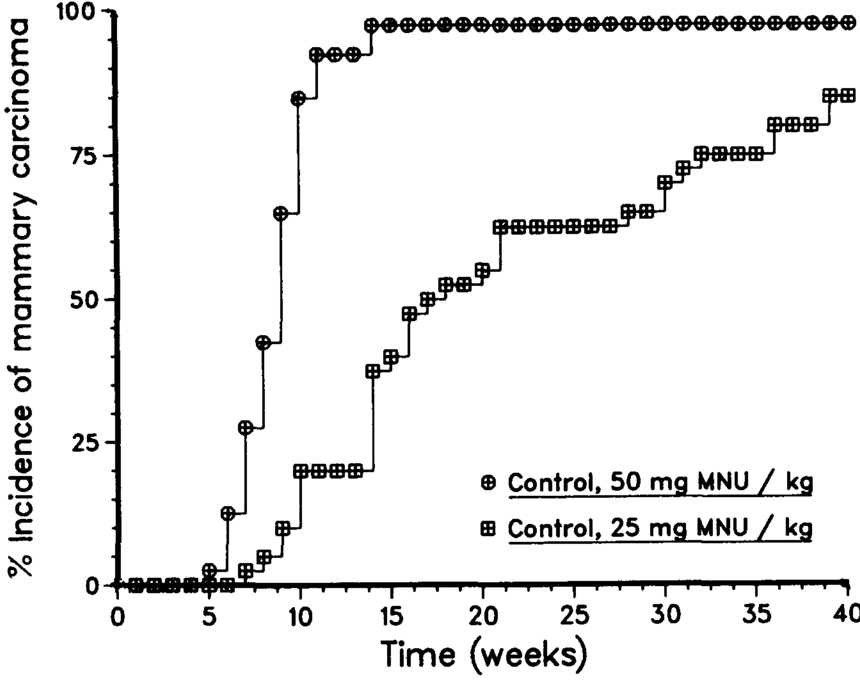

FIG. 6. Increase in mammary carcinoma incidence (%) of SD-rats induced with MNU.

tumor incidence was seen as compared to a 30% difference following the optimal treatment of animals induced with the high dose of MNU. The low dose of TLP, however, did not effect any tumor growth suppression.

DISCUSSION

Alkyl lysophospholipids belong to a new class of antineoplastic agents that seem to combine cytotoxic and immunological mechanisms of action. However, both activities are observed at different dose ranges. The "immunomodulatory" activity is exerted at low dosages, probably activating macrophages and/or natural killer cells (17). Concomitantly, significant enlargement of the spleen is detected whereas high dosages considerably reduce the spleen size (unpublished data).

In the present paper, the values of both anticancer mechanisms of action were investigated regarding their

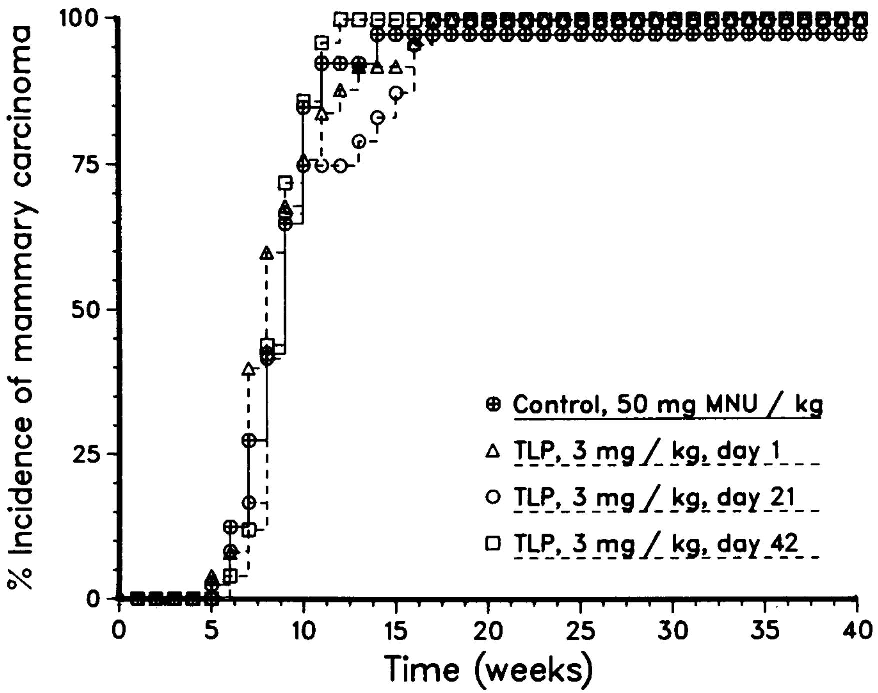

FIG. 7. Influence of TLP (3 mg/kg) on the increase in mammary carcinoma incidence of SD-rats induced with 50 mg/kg MNU.

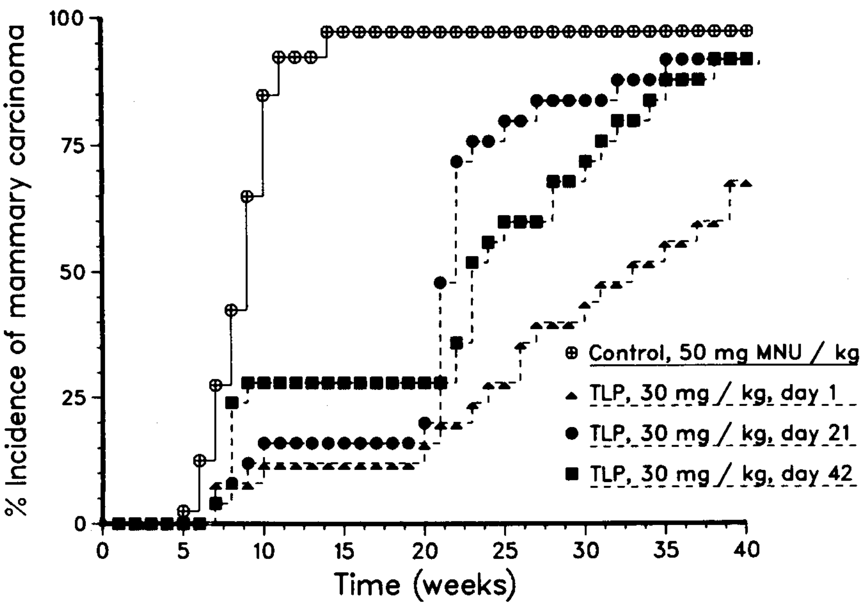

FIG. 8. Influence of TLP (30 mg/kg) on the increase in mammary carcinoma incidence of SD-rats induced with 50 mg/kg MNU.

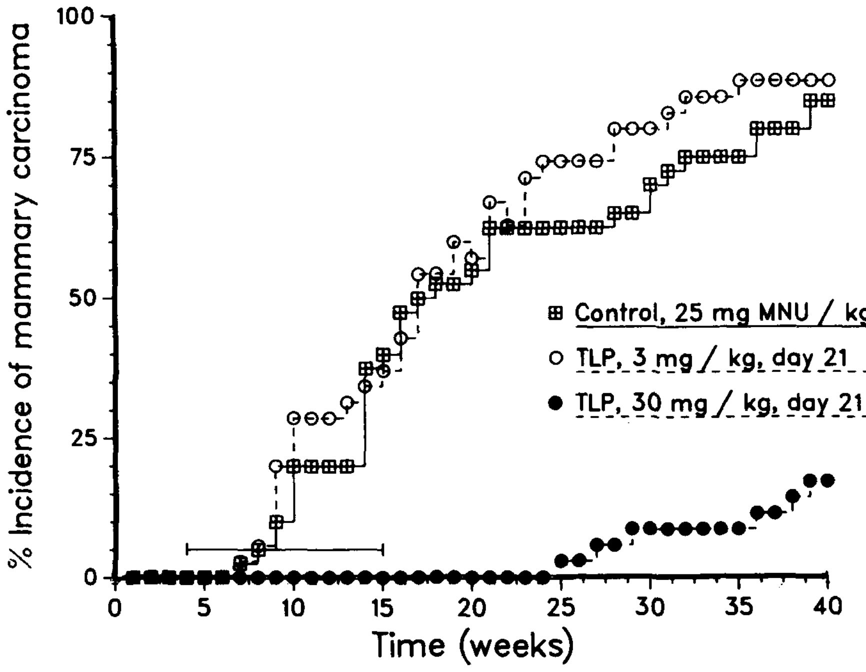

FIG. 9. Influence of TLP (3 or 30 mg/kg) on the increase in mammary carcinoma incidence of SD-rats induced with 25 mg/kg MNU (|——|): duration of TLP-administration).

modulatory efficacy on the carcinogenic effect of two different carcinogens. Low doses of the alkyl lysophospholipid TLP were ineffective in suppressing the formation of MNU-induced tumors. This is in accordance with the results of Berdel et al. (18), who administered 50 μg ET-18-OCH_3/mouse equivalent to 2.5 mg/kg and failed to modulate the occurrence of leukemias in genetically determined AKR mice or in C57Bl/6 mice that had been irradiated to induce lymphomas. It differs from the results of Munder et al. (9), who administered the same dose of ET-18-OCH_3 to 20-methylcholanthrene-induced Balb C mice and observed significant reduction of tumor incidence. However, the administration of higher doses of either compound was associated with significant inhibition of rat mammary tumor occurrence. It is interesting to note that the effect following TLP was pronounced more as compared with the respective control when the lower dose of MNU was used (25 instead of 50 mg/kg).

In preceding experiments on the therapeutic efficacy of ET-18-OCH_3 and TLP, both agents exhibited antineoplastic activities against established mammary lesions when sufficiently high doses were used (11).

However, ET-18-OCH_3 did not prolong the life span of LE-rats bearing overt leukemia (unpublished data). Since the manifestation of both types of tumors was at least partly suppressed when the onset of administration lie between induction and manifestation of tumors, different mechanisms of action might be involved.

Unlike various conventional cytotoxic agents, alkyl lysophospholipids do not affect DNA (19); therefore, they could be used for cancer prevention. Only compounds without irreversible or severe side effects are suited for the prevention of cancer, since the onset of administration would lie before any visible cancer growth and would last over a considerable period of time. Our results indicate that alkyl lysophospholipids act between the time of initiation and the time of manifestation of tumors. Consequently, a treatment following an exposure to certain carcinogens might be recommended.

In summary, the observed modulation of chemically induced carcinogenesis is promising, and it should be the basis for studies on the presently unknown basic mechanisms of action.

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Antineoplastic Activity of Conjugates of Lipids and 1-β-D-Arabinofuranosylcytosine[1]

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Five different lipid conjugates of 1-β-D-arabinofuranosylcytosine (ARA-C) were tested in comparison with ARA-C, the ether lipid ET-18-OCH₃ (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) and their equimolar mixtures. The compounds were tested in vitro for cytotoxicity in the trypan blue dye exclusion test with cells from six different leukemias, one glioblastoma and two bronchogenic carcinomas of human origin. The compounds were given in vivo to assess their therapeutic activity against 3-Lewis lung carcinoma (3-LL) of syngeneic $C_{57}Bl_6$ mice. Although some of the conjugates have shown cytotoxic activity in vitro against the cell samples tested, they have not revealed higher cytotoxicity than ET-18-OCH₃, ARA-C or their equimolar mixtures. In these experiments, ARA-CDP-D,L-MBA was the conjugate with the highest cytotoxicity. Some of the conjugates significantly inhibited tumor growth and also increased survival of $C_{57}Bl_6$ mice with intraperitoneally (ip) implanted 3-LL. In these experiments, ARA-CDP-D,L-PTBA, ARA-CDP-D,L-PBA, ARA-CDP-L-dipalmitin and ARA-CDP-D,L-PCA were more active than either the parent compounds ARA-C and ET-18-OCH₃ alone or their equimolar mixtures. Furthermore, when the conjugates were injected as adjuvant chemotherapy shortly after the surgical removal of the primary 3-LL, they inhibited the metastasis of 3-LL to the lungs of the animals, demonstrated by an increase of the survival time and the number of surviving animals. The mode of action of these new antineoplastic compounds still is speculative.
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Recently, there is an increasing interest in the antineoplastic properties of certain ether lipids and analogs. This subject has been reviewed in detail (1). Among other modes of action, some of those compounds exert direct cytotoxicity during incubation with neoplastic cells (1). Since favorable activity of 1-β-D-arabinofuranosylcytosine (ARA-C) conjugates of nontoxic ester-linked phospholipids was found in experimental leukemia and myeloma (2,3), we tried to improve these results by synthesizing and testing ARA-C conjugates of a variety of cytotoxic ether lipids (4–6). Here we report on the antineoplastic in vitro and in vivo efficacy of five lipid conjugates of ARA-C, including four conjugates of either lipids with a 1-*O*-alkyl- or a 1-S-alkyl linkage in the *sn*-1 position of the lipid moiety.

MATERIALS AND METHODS

Drugs. The chemical structures of the conjugates tested are depicted in Figure 1. Details on synthesis, chemical properties and preliminary preclinical toxicity data have been published (5,6). The chemical structures of the parent compounds, including the ether lipids 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH₃) and BM 41.440 also have been published (5,6). The compounds were added to 10% serum-containing culture medium for the in vitro assays and were added to saline for the treatment experiments in vivo.

In vitro assays for antineoplastic activity. Cell cultures and cytotoxicity assays were performed as recently described (7). In the trypan blue dye exclusion assay, cells were incubated with the test compounds for various times in serum-containing medium and cytotoxic action of the drugs was measured as an increase of trypan blue uptake into the cells given as percentage of the controls.

3-Lewis-lung experiments in vivo. The tumor model has been used as described recently (8,9). Briefly, 3-LL was

TABLE 1

Influence of 2-Lysophosphatidylcholine (2-LPC), ET-18-OCH₃, ARA-C, an Equimolar Mixture of ARA-C and ET-18-OCH₃ and the Conjugate ARA-CDP-D,L-MBA on the Trypan Blue Dye Exclusion of HL 60-Leukemic Cell Line In Vitro

		Trypan blue dye exclusion after the following incubation times	
Compound	Concentration[a]	24 hr	48 hr
2-LPC	20	108[b]	117
ET-18-OCH₃	1	91	55
	10	18	0
	20	6	0
ARA-C	0,45[a]	75	34
	4,5	67	19
	9	65	25
Conjugate (ARA-CDP-D,L-MBA)	1,45[a]	88	93
	14,5	80	41
	29	67	27
Equimolar mixture[c]	a,c	84	26
		20	0
		6	0

[a]The concentrations of 2-LPC and ET-18-OCH₃ are given in μg/ml. ARA-C and the conjugate were incubated in equimolar concentrations to ET-18-OCH₃ (μg/ml).
[b]Values are given as percentage of the control.
[c]1:1 mixture of ET-18-OCH₃ and ARA-C in equimolar concentrations to the conjugate.

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studied in syngeneic $C_{57}Bl_6$ mice that were kept under standard conditions. For experiments regarding primary tumor growth, 3-LL cells were given intraperitoneally (ip) at day 0. Ip treatment was performed on days 1–5. Survival was determined at day 20 and after ≥60 days. Death from a tumor occurred within 40 days after the tumor transplantation. For metastasis experiments, 10^6 3-LL cells were given subcutaneously into the foot pad at day 0. The tumor was removed surgically at a diameter of 0.6 cm. Treatment was given ip after randomization on days 1–5 after operation. The end-point of the experiments was either death from metastasis or survival. Survival was determined at days 30, 40 and after >60 days.

Statistical analysis. This was performed with the 2 × 2 contingency table (two-tailed test) and p-values were obtained as published (10).

RESULTS

In vitro. The compounds were tested in vitro for cytotoxicity using the trypan blue dye exclusion test in cells from six different leukemias, one glioblastoma and two bronchogenic carcinomas of human origin. Although the conjugates have shown cytotoxic activity against the cell samples tested, they have not revealed higher cytotoxicity than ET-18-OCH_3, ARA-C or their equimolar mixtures. In these experiments, the LC_{50} values (LC_{50} = lethal concentration for 50% of the cells) for ET-18-OCH_3 after 48 hr of incubation were found to be <3 μM for the leukemias and ca. 20 μM for the solid tumors. The order of efficacy against leukemias was ARA-C > equimolar mixtures > ET-18-OCH_3 > conjugates (ARA-CDP-D,L-MBA). The order of efficacy against the solid tumors was ET-18-OCH_3 > equimolar mixtures > conjugates (ARA-CDP-D,L-MBA) > ARA-C. ARA-CDP-D,L-MBA was found to be the conjugate with the highest cytotoxicity in vitro. Table 1 summarizes the results for the human leukemic cell line HL 60 as an example. More details and the exact LC_{50} values for the other cell lines tested recently have been published (5).

3-Lewis lung carcinoma in vivo. Table 2 summarizes our experiments testing the therapeutic efficacy of the conjugates, an equimolar (1:1) mixture of ARA-C and ET-18-OCH_3 at the same dose level and the parent compounds ARA-C and ET-18-OCH_3 alone on the primary 3-LL growth. Similar to previous experiments in L1210

ara-CDP-L-dipalmitin

ara-CDP-DL-PBA

ara-CDP-DL-PCA

ara-CDP-DL-MBA

ara-CDP-DL-PTBA

FIG. 1. Chemical structures of the conjugates of lipids and 1-β-D-arabinofuranosylcytosine (ARA-C).

TABLE 2

3-LL Tumor Growth under the Influence of Different Drugs and Treatment Schedules[a]

Tumor burden (3-LL cells)	Treatment groups	Survivors/total 20 days	χ^2	p[b]	Survivors/total ≥60 days	χ^2	p
1×10^5	Control (PBS)	4/10			1/10		
	Ara-CDP-D,L-MBA 100 mg/kg/day	8/10	3.33	0.069	2/10	0.39	0.527
	Ara-CDP-D,L-PBA 100 mg/kg/day	10/10	8.57	0.003	10/10	16.36	<0.001
	Ara-CDP-L-Dipalmitin 100 mg/kg/day	5/5	5.0	0.025	5/5	11.25	<0.001
	Ara-C 100 mg/m²/day	9/9	7.89	0.005	7/9	8.93	0.002
	Ara-C 200 mg/kg/day	10/10	8.57	0.003	8/10	9.90	0.001
	ET-18-OCH_3 5 mg/kg/day	6/10	0.80	0.371	3/10	1.25	0.254
	ET-18-OCH_3 20 mg/kg/day	9/10	5.49	0.019	2/10	0.39	0.527
5×10^5	Control (PBS)	3/25			0/25		
	Ara-CDP-D,L-PBA 100 mg/kg/day	18/19	29.62	<0.001	6/19	9.14	0.002
	Ara-CDP-L-Dipalmitin 100 mg/kg/day	5/5	16.50	<0.001	2/5	10.71	<0.001
	Ara-CDP-D,L-PCA 100 mg/kg/day	5/5	16.50	<0.001	1/5	5.17	0.022
	Ara-CDP-D,L-PTBA 80 mg/kg/day	9/10	19.29	<0.001	6/10	18.10	<0.001
	Equimolar mixture[c] (Ara-C/ET-18-OCH_3)	6/14	4.81	0.028	0/14		ns[b]

[a]3-LL cells were given ip at day 0; ip treatment was performed on days 1-5; survival was determined at day 20 and after ≥60 days.
[b]Significance level in 2 × 2 contingency analysis (two-tailed test) was compared to the control group with identical tumor burden; ns = not significant.
[c]1:1 mixture on a molar basis of both compounds with a concentration of Ara-C and ET-18-OCH_3 equimolar to the conjugate solution used for parallel experiments.

TABLE 3

3-LL Metastasis under the Influence of Different Drugs and Treatment Schedules[a]

Treatment groups	Survivors/total 30 days	χ^2	p[b]	40 days	χ^2	p	>60 days	χ^2	p
Control (PBS)	22/28			14/28			9/28		
Ara-CDP-D,L-PBA 100 mg/kg/day	24/24	5.81	0.016	18/24	3.41	0.065	11/24	1.02	0.317
Ara-CDP-D,L-PTBA 80 mg/kg/day	25/25	6.04	0.014	18/25	2.67	0.100	15/25	4.14	0.042
Equimolar mixture[c] (Ara-C/ET-18-OCH_3)	1/5	6.89	0.008*	1/5	1.54	0.220	1/5	0.30	0.583

[a]10^6 3-LL cells were given subcutaneously into the footpad at day 0; the tumor was surgically removed at a diameter of 0.6 cm; treatment was given ip on days 1-5 after the operation. The end point of the experiment was either death from metastasis or survival. Survival was determined at days 30 and 40 and after >60 days.
[b]Significance level in 2 × 2 contingency analysis, two-tailed test.
*Values indicate experiments in which the outcome of control was superior to the treatment group.
[c]See Table 2.

leukemia (5,6), 80–100 mg/kg/day given for five consecutive days proved to be optimal for the conjugates; higher doses started to produce toxicity. ARA-C dose levels and treatment schedules were chosen according to National Cancer Institute protocols (11) with some modifications. Doses up to 200 mg/kg/day of ARA-C given for five consecutive days were nontoxic for healthy mice. The ET-18-OCH_3 was given at a dose range of 5–20 mg/kg/day, using previous experience as a guideline (8,9). ARA-CDP-D,L-PBA and ARA-CDP-D,L-PTBA and ARA-CDP-L-Dipalmitin revealed the strongest therapeutic efficacy, leading to a significant increase of surviving animals up to a tumor load of 5×10^5 3-LL-cells and to an increase of survival time of the treated mice even with a tumor load of 10^6 3-LL-cells. Interestingly, the ARA-CDP-D,L-MBA was less effective. Systemic therapeutic efficacy on 3-LL metastasis was tested; conjugates of ether lipiuds and ARA-C were given as adjuvant chemotherapy shortly after the surgical removal of the primary tumor. In these experiments, ARA-CDP-D,L-PBA and ARA-CDP-D,L-PTBA showed a moderate therapeutic activity measured as an increase of survival time and absolute numbers of surviving animals, which was superior to equimolar mixtures of ARA-C and ET-18-OCH_3. Table 3 summarizes some of these experiments.

DISCUSSION

Among various conjugates of lipids and 1-β-D-arabinofuranosylcytosine (ARA-C), the thioether lipid conjugate ARA-CDP-D,L-PTBA and the 1-O-alkyl lipid conjugate ARA-CDP-D,L-PBA showed therapeutic efficacy against the 3-LL primary tumor growth and its metastasis to the lungs of syngeneic $C_{57}Bl_6$ mice. Both conjugates were superior to ARA-C and ET-18-OCH_3 by themselves or their equimolar mixtures at the same dose level. Effective therapeutic regimens were relatively well-tolerated by the mice. The activity of the conjugates found in the 3-LL model followed prior observations, which described therapeutic effects of these compound in mouse leukemias L1210 and P388 (4–6).

Interestingly, this in vivo activity does not correlate directly with in vitro cytotoxicity because the ARA-CDP-D,L-MBA, which is one of the most active conjugates in vitro with regard to direct cytotoxicity, produced only minor therapeutic effects in vivo. Thus, further experiments are necessary to elucidate both the metabolism and the mode of conjugate action. However, the hypothesis leading to these conjugates, which is to produce a drug that might release two cytotoxic metabolites with different cytotoxicity targets, remains speculative. In conclusion, the comparatively high therapeutic activity of the two ether lipid conjugates of ARA-C, ARA-CDP-D,L-PTBA and ARA-CDP-D,L-PBA in the 3-LL and leukemias in mice is a strong incentive to further investigate these compounds as experimental drugs for cancer treatment within a wider preclinical test program.

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Synthesis of Thioether Phosphocholine Analogues[1]

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The synthesis of thioether phospholipids, which represent a new class of antitumor agents, is reported here. In particular, the route of synthesis of 3-hexadecylmercapto-2-methoxymethylpropyl-2′-trimethylammonio-ethyl phosphate (BM 41.440, Ilmofosine), one of the most potent cytostatic/cytotoxic derivatives, is described in detail. Starting with diethyl *bis*-hydroxymethylmalonate, ethyl 2-phenyl-1,3-dioxane-5-carboxylate is formed via diethyl 2-phenyl-1,3-dioxane-5,5-dicarboxylate and 5-ethoxycarbonyl-2-phenyl-1,3-dioxane-5-carboxylic acid. Reduction of ethyl 2-phenyl-1,3-dioxane-5-carboxylate with $LiAlH_4$ affords 5-hydroxymethyl-2-phenyl-1,3-dioxane. Alkylation with dimethyl sulfate gives 5-methoxymethyl-2-phenyl-1,3-dioxane. The ring structure then is opened by *N*-bromosuccinimide, resulting in the formation of 3-bromo-2-methoxymethylpropyl benzoate. Reaction of 3-bromo-2-methoxymethylpropyl benzoate with the sodium salt of hexadecanethiol leads to 3-hexadecylmercapto-2-methoxymethylpropanol, which is reacted with a cyclic chlorophosphate to give the corresponding phosphorylated 3-hexadecylmercapto-2-methoxymethylpropanol. Treatment with trimethylamine yields BM 41.440. This compound already has been tested in clinical phase I/II trials in West Germany.
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Ether phospholipids are among the most potent biologically active phospholipids (1,2) and have been shown to be required for a series of vitally important physiological processes such as platelet activation (2), vasodilation (3) and chemotaxis (4). During recent years, synthetic thioether phosphocholine analogues have gained increasing interest due to their antitumor activity in a variety of tumor models in vitro and in vivo (5-9). Furthermore, the implication of platelet-activating factor (PAF), which has been identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (10,11) as a primary mediator of acute inflammatory and allergic reactions (12), has stimulated great interest, particularly in analogues similar in structure to biologically active PAF (13,14). 3-hexadecylmercapto-2-methoxymethylpropyl 2′-trimethylammonio-ethyl phosphate (BM 41.440) (prop. INN: Ilmofosine) is one of the most potent antineoplastic thioether phosphocholine analogues reported so far (5-7,15). This compound recently has been tested in clinical phase I/II trials in refractory cancer patients in West Germany (16). This report describes general synthetic routes to thioether choline phospholipids (Scheme 1) and, in particular, the synthesis of BM 41.440 (Scheme 2[8]).

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Abbreviations: BM 41.440, 3-hexadecylmercapto-2-methoxymethylpropyl-2′-trimethylammonio-ethyl phosphate; DMF, dimethyl formamide; NBS, *N*-bromosuccinimid; PAF, platelet activating factor.

EXPERIMENTAL

Diethyl 2-phenyl-1,3-dioxane-5,5-dicarboxylate (Scheme 2 [1]). Twenty-two g (0.1 mol) of diethyl *bis*-hydroxymethylmalonate (Aldrich) in 200 ml toluene is refluxed with 10.6 g (0.1 mol) of benzaldehyde and 0.2 g *p*-toluenesulfonic acid for three hr using a Dean Stark trap. The toluene then is evaporated in vacuo and the residue purified by distillation to afford 26.6 g (86%); $bp_{1.1}$ 167-170 C.

5-Ethoxycarbonyl-2-phenyl-1,3-dioxane-5-carboxylic acid (Scheme 2[2]). To 4 g (0.072 mol) of potassium hydroxide in 100 ml ethanol, 15.4 g (0.05 mol) of Scheme 2[1] is added. After stirring for four hr, the ethanol is evaporated in vacuo and the residue treated with 72 ml 1 N hydrochloric acid with cooling. The precipitate is filtered off by suction; yield: 13.0 g (93%); mp 111-113 C.

Ethyl 2-phenyl-1,3-dioxane-5-carboxylate (Scheme 2[3]). A mixture of 16.2 g (0.058 mol) of Scheme 2[2] and 0.1 ml piperidine in 30 ml absolute pyridine are heated under reflux for five hr. After cooling, the solution is poured onto 30 ml ice-cold 12 N hydrochloric acid. The solution is extracted twice with dichloromethane, and the combined organic layer is dried and evaporated in vacuo; yield: 13.4 g (98%); mp 68-71 C.

5-Hydroxymethyl-2-phenyl-1,3-dioxane (Scheme 2[4]). The mixture of 11.53 g (0.049 mol) of Scheme 2[3] and 1.05 g (0.028 mol) of $LiAlH_4$ in 100 ml absolute diethyl ether is stirred for 3.5 hr at room temperature. The slurry is worked up under ice, cooling by subsequently adding 8 ml ethyl acetate, 4 ml water, 4 ml 15% sodium hydroxide solution and 12 ml of water. The ethereal layer is separated, dried and evaporated in vacuo to afford 9.17 g (96%) of a pale-yellow oil.

5-Methoxymethyl-2-phenyl-1,3-dioxane (Scheme 2[5]). Seven ml (0.073 mol) of dimethyl sulfate is added dropwise to a vigorously stirred mixture of 9.17 g (0.047 mol) of Scheme 2[4] in 50 ml dichloromethane and 0.5 g tetrabutylammonium iodide. After stirring for 18 hr, 10 ml conc. ammonia and 40 ml water are added. The solution is extracted three times with 60 ml dichloromethane. The combined organic layer is washed consecutively with 15 ml 2 N hydrochloric acid and water until neutral. The organic layer is dried and evaporated in vacuo. Distillation of the residue yields 9.1 g (93%), $bp_{0.1}$ 117-120 C.

3-Bromo-2-methoxymethylpropyl benzoate (Scheme 2 [6]). A mixture of 9.4 g (0.045 mol) of Scheme 2[5], 8.8 g (0.049 mol) of *N*-bromosuccinimide and 1 g barium carbonate in 100 ml dichloromethane are refluxed for 2.5 hr. After cooling, the reaction mixture is evaporated in vacuo, the residue triturated with hexane, filtered and the filtrate evaporated in vacuo to afford 12.9 g of a light-yellow oil. The oil is used without further purification in the reaction.

3-Hexadecylmercapto-2-methoxymethylpropanol (Scheme 2[7]). The oil residue of the foregoing reaction, 12.9 g of Scheme 2[6], in 35 ml ethanol is added to a prepared solution of 1.15 g Na (0.05 mol) in 40 ml ethanol and 12.9 g (0.05 mol) hexadecanthiol in 40 ml ethanol and stirred for 20 hr at room temperature. The mixture is acidified with hydrochloric acid/ethanol and evaporated

Ⓐ

$CO_2C_2H_5$ / R_1-CH / $CO_2C_2H_5$ [1a] → CH_2OH / R_1-CH / CH_2OH [2a]

Ⓑ

CH_2OH / $HO-CH$ / CH_2OH [1b] → CH_2-O / $HO-CH$ / CH_2-O (Ph) [2b]

[2a], [2b] → CH_2-O / $R_1-(O)-CH$ / CH_2-O (Ph) [3] →

CH_2-Br / $R_1-(O)-CH$ / $CH_2-O-C(=O)-Ph$ [4] → CH_2-S-R_2 / $R_1-(O)-CH$ / CH_2-OH [5] →

CH_2-S-R_2 / $R_1-(O)-CH$ / $CH_2-O-P(=O)(-O-CH_2CH_2-O-)$ [6] → CH_2-S-R_2 / $R_1-(O)-CH$ / $CH_2-O-P(=O)(O^{\ominus})-O-(CH_2)_2-\overset{\oplus}{N}(CH_3)_3$ [7]

SCHEME 1. General routes of synthesis of ether phospholipid derivatives.

$$(HOCH_2)_2C(CO_2C_2H_5)_2 \xrightarrow{PhCHO} [1] \xrightarrow[2.\ HCl]{1.\ KOH} [2] \xrightarrow{\Delta} [3] \xrightarrow{LiAlH_4} [4] \xrightarrow{Me_2SO_4} [5] \xrightarrow{NBS} [6] \xrightarrow[2.\ HCl/C_2H_5OH]{1.\ C_{16}H_{33}SNa} [7] \rightarrow [8]$$

[1] 2-Phenyl-1,3-dioxane-5,5-di($CO_2C_2H_5$)

[2] 2-Phenyl-1,3-dioxane-5-($CO_2C_2H_5$)-5-(CO_2H)

[3] 2-Phenyl-1,3-dioxane-5-$CO_2C_2H_5$

[4] 2-Phenyl-1,3-dioxane-5-CH_2OH

[5] 2-Phenyl-1,3-dioxane-5-CH_2OCH_3

[6] CH_2-Br / H-C-CH_2-OCH_3 / CH_2-O-C(=O)-Ph

[7] CH_2-S-$C_{16}H_{33}$ / H-C-CH_2-OCH_3 / CH_2-OH

Reagent for [7] → [8]: Cl-P(=O) cyclic (2-chloro-2-oxo-1,3,2-dioxaphospholane), then $(CH_3)_3N$

[8] CH_2-S-$C_{16}H_{33}$ / H-C-CH_2-OCH_3 / CH_2-O-P(=O)(O^-)-O-CH_2-CH_2-$N^+(CH_3)_3$

SCHEME 2. Route of synthesis of BM 41.440.

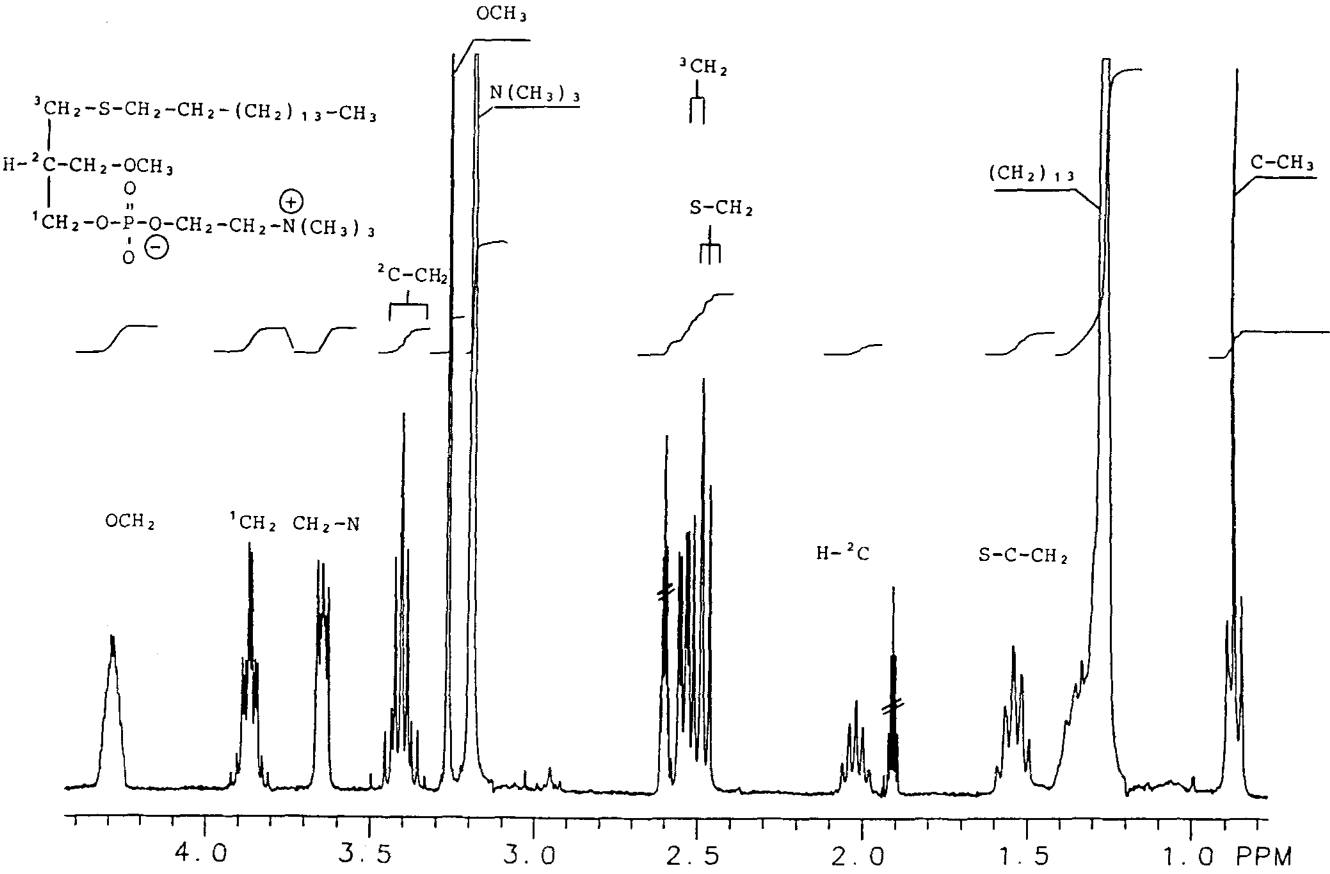

FIG. 1. ¹H-NMR spectrum of BM 41.440 in Me_2SO-d_6.

in vacuo. The residue is taken up in water and extracted three times with dichloromethane. The combined organic layer is treated with $NaHCO_3$ and water, dried and evaporated in vacuo. Most of the ethyl benzoate formed is distilled off in vacuo and the residue purified by silica gel (150 g) chromatography using diethyl ether/hexane (1:5, v/v) as eluant; yield: 10.7 g (66%); mp 42-44 C.

3-Hexadecylmercapto-2-methoxymethylpropyl 2'-trimethylammonio-ethyl phosphate (Scheme 2[8] = BM 41.440). A solutoin of 14.3 g (0.1 mol) of 2-chloro-2-oxo-1,3,2-dioxaphospholane in 50 ml absolute dichloromethane is added drop-wise with stirring to a solution of 21.2 g (0.059 mol) of Scheme 2[7] and 23 ml triethylamine in 170 ml absolute dichloromethane while the temperature is kept below −25 C. After 30 min, the reaction mixture is evaporated in vacuo at a bath temperature of 35 C; the residue is treated with 250 ml diethyl ether and filtered by suction. The filtrate is evaporated in vacuo and the residue dissolved in 200 ml absolute acetonitrile saturated with trimethylamine. The solution is stirred at room temperature for four days, the precipitate formed is filtered off by suction and consecutively washed with 30 ml absolute acetonitrile and 30 ml absolute acetone. The crude material, 25.6 g, is dissolved in 12.5 ml dichloromethane, filtered; then 50 ml acetone is added at 10 C with stirring. After one hr at 10 C, the precipitate is filtered off by suction and dried over phosphorus pentoxide; yield: 15.7 g (46%). The product contains 3 mol of water.

RESULTS AND DISCUSSION

Depending on the nature of the final product, the synthesis of thioether phospholipid analogues starts either with a malonate derivative (Scheme 1[1a]) or with glycerol (Scheme 1[1b]). Scheme 1 shows two routes, A and B, which are representative for the synthesis of most of the thioether phospholipids.

Route A: the substituent R_1 in the diethyl malonate (Scheme 1[1a]) stands for an alkyl, benzyl or phenyl group that may be substituted again by other groups/atoms. Such substituted diethyl malonates either are available commercially or can be synthesized easily. Reduction of Scheme 1[1a] by $LiAlH_4$ affords the corresponding propane-1,3-diol (Scheme 1[2a]) usually in yields of 80-90%. The following ring closure of Scheme 1[2a] to a 1,3-dioxane derivative (Scheme 1[3]) is performed with benzaldehyde (PhCHO) in the presence of *p*-toluenesulfonic acid. The sequence of the last two reactions also can be reversed.

Route B: starting with glycerol (Scheme 1[1b]), 1,3-benzylidene glycerol (Scheme 1[2b]) is formed in yields of about 50% by reaction with benzaldehyde in the presence of gaseous hydrochloric acid (17). The sodium salt of Scheme 1[2b], provided by sodium hydride in toluene or dimethyl formamide (DMF), is converted to a 2-alkoxy-1,3-benzylidene glycerol (Scheme 1[3]) by reaction with an alkyl halide or a dialkyl sulfate.

The dioxane ring of Scheme 1[3] is opened by *N*-bromosuccinimid (NBS) in dichloromethane, resulting in

the formation of a benzoylated propyl bromide derivative (Scheme 1[4]) (18,19). This intermediate usually is not purified. Intermediate (Scheme 1[4]) then reacts with the sodium salt of a long-chain thiol (RS-Na) to form a 3-alkylmercaptopropanol (Scheme 1[5]) with ethyl benzoate as side-product. The yield for the last two reaction steps is about 60–70%. The reaction of benzoate as side-product. The yield for the last two reaction steps is about 60–70%. The reaction of Scheme 1[5] with a cyclic chlorophosphate in the presence of a base-like triethylamine gives the corresponding phosphorylated 3-alkylmercaptc propanol (Scheme 1[6] (20,21). The cyclic phosphate o Scheme 1[6] is split by trimethylamine ($(CH_3)_3N$; 20,21] The last two steps usually afford acceptable yields (40–75%) of the desired thioether phosphocholine analogues (Scheme 1[7]).

In some cases, the final product crystallizes after standing for three or four days and is sufficiently pure. On the other hand, some of the analogues have to be purified by silica gel chromatography using dichloromethane/methanol/water (65:25:4) (27) as eluant. Scheme 2 illustrates as an example for thioether phospholipids the synthesis of BM 41.440. Details are given in the experimental section. The structures of intermediates and end products were confirmed by ^{1}H-NMR spectroscopy and/or mass spectrometry. The purity was established by elementary analysis. As an example, the ^{1}H-NMR spectrum (Varian XL-300) of BM 41.440 is given in Figure 1.

In conclusion, the synthetic pathways as outlined in Scheme 1 have a great deal of flexibility, providing a convenient general method for the preparation of a wide scope of structurally related thioether phospholipid analogues suitable for structural, chemical, enzymological and therapeutic studies.

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Pharmacokinetics of the Thioether Phospholipid Analogue BM 41.440 in Rats[1]

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BM 41.440 (1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine) is a cytotoxic thioether phospholipid analogue that recently has entered phase I trials in cancer patients. The objective of this study was to evaluate the pharmacokinetics of this compound in female rats after administration of a single oral dose (15 mg/kg body weight [bw]). Furthermore, BM 41.440 serum concentrations were determined under a daily oral treatment of up to 13 weeks. Blood samples were obtained via permanent catheters from the femoral arteries before and after drug administration for a total of 120 hr. Urine was collected in 24 hr-intervals for 120 hr; the volume was measured, and aliquots were stored at −20 C until analytical determination of the thioether derivative. BM 41.440 was assayed in serum and urine by means of a specific, newly developed reverse-phase high pressure liquid chromatography technique. Mean maximum serum concentrations (1.7 μg/ml, n = 4 animals) were attained after seven hr. A terminal half-life of ca. 27 hr was calculated from the rate constant for the terminal elimination phase ($\lambda_z \sim 0.026$/hr). The mean serum BM 41.440 concentration-time-area-under-the-curve was 52.9 mg × hr/l. The ratio of total body clearance to absorption fraction was 4.7 ml/min × kg bw. Only a small amount of the drug was found in the urine. The quantity excreted in the urine during a 24 hr-interval never exceeded 1.5% of the administered dose. Under a daily oral schedule (15 mg/kg bw × day) up to 13 weeks, mean BM 41.440 serum concentrations of 3.3 ± 0.5 μg/ml and 5.2 ± 1.2 μg/ml (mean ± S.D., n = 10 animals) were found after five and 13 weeks, respectively. Taken together, the data indicate that BM 41.440 was absorbed from the gastrointestinal tract after oral administration and that accumulation of BM 41.440 can occur in rats.
Lipids 22, 952–954 (1987).

Alkyl lysophospholipids (ALP) are analogues of lysophosphatidylcholine, which is a minor but important intermediate in the continuous exchange and renewal of phospholipids in cellular membranes via the deacylation/reacylation cycle (Lands pathway) (1,2). ALP represent a new class of antitumor agents (3-6). The prophylactic and therapeutic, antineoplastic, antimetastatic and anti-invasive effects of ALP have been demonstrated in different murine and rat tumor models (3-8), including autochthonous neoplasms (9) and different transplantation tumors (10,11).

The antitumor activity of ALP has been explained by a direct and selective cytotoxic action on neoplastic cells and by the generation of tumoricidal, immuno-competent cells of the monocyte/macrophage lineage in vitro and in vivo (3-5,12). In addition, the induction of a differentiation process in human and murine myeloid leukemia cells has been described (13).

The molecular mechanism whereby these compounds exert their direct antineoplastic effect is related to a possibly specific interference with the phospholipid metabolism of neoplastic cells (12-15), although the precise mode of action is not explained completely yet.

This investigation deals with the pharmacokinetic behavior of cytotoxic 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440, prop. INN: Ilmofosine) in rats (Scheme 1). It reports the first pharmacokinetic study performed with a nonradiolabeled ether lipid analogue, thus providing information about the various kinetic parameters for the first time. Pharmacokinetics with a radiolabeled phospholipid analogue closely related in structure to BM 41.440 have been reported by our group (16) and others (17).

MATERIALS AND METHODS

BM 41.440 was supplied as a white, crystalline powder by Boehringer Mannheim GmbH (Mannheim, FRG).

Concentrations of the thioether phospholipid analogue were determined in serum and urine of fasted Sprague-Dawley rats (260-380 g body weight [bw]) after administration of a single oral dose of 15 mg/kg bw. Furthermore, BM 41.440 serum and urine concentrations were evaluated under a daily oral schedule of 15 mg/kg bw after five and 13 weeks, respectively. For administration by gavage, the drug was dissolved in a 0.5% methyl cellulose solution (Tylose, Fluka AG, Buchs, Switzerland). Samples of arterial blood (1 ml) were collected through permanent catheters placed in the femoral arteries at the following times after BM 41.440 administration: 0, 0.5, 1, 2, 3, 5, 7, 24, 31, 48, 55, 72, 96 and 120 hr. Excessive blood loss of each animal was avoided by dividing all animals investigated into four groups of four rats each and by withdrawing blood from animals of only one group

$$
\begin{array}{l}
\qquad\qquad\quad H_2C-S-C_{16}H_{33} \\
\qquad\qquad\qquad | \\
CH_3-O-CH_2-CH \qquad O \\
\qquad\qquad\qquad | \qquad\quad \| \\
\qquad\qquad\quad H_2C-O-P-O-(CH_2)_2-\overset{\oplus}{N}(CH_3)_3 \\
\qquad\qquad\qquad\qquad\quad | \\
\qquad\qquad\qquad\qquad\quad O^{\ominus}
\end{array}
$$

SCHEME 1. Chemical structure of 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine, BM 41.440.

[1]Presented at the symposium on "Ether Lipids in Oncology," Göttingen, Federal Republic of Germany, December 1986.

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Abbreviations: ALP, Alkyl lysophospholipid analogue(s); BM 41.440, 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine; HPLC, high pressure liquid chromatography.

at each time indicated between 0 and 120 hr. Then, all samples for each time-point of blood withdrawal were pooled. Upon collection, blood samples were kept on ice and immediately centrifuged at 4 C for 10 min at 2000 rpm. Serum was decanted and stored at −20 C until analysis.

Urine was collected in 24 hr-intervals for 120 hr, the volume was measured and aliquots were stored frozen at −20 C until analytical determination of BM 41.440.

BM 41.440 was assayed in serum and urine by means of a specific, newly developed reverse-phase high pressure liquid chromatography (HPLC) technique. To 1 ml serum in a 10 ml glass centrifuge tube, 0.1 ml internal standard (BM 41.448; Boehringer Mannheim GmbH) was added, mixed and the mixture was lyophilized. The dry residue was extracted first with 2 ml, then 1 ml $CHCl_3/CH_3OH$ (2:1, v/v), using a whirlmix and an ultrasonic bath (for two min) and centrifuged at 3000 × g for five min. The combined supernatants were applied onto a disposable silica cartridge (Analytichem Int. [Frankfurt, FRG]) preconditioned with 4 ml CH_3OH/CH_2Cl_2 (1:1, v/v) and 1 ml CH_3OH/glacial acetic acid (9:1, v/v). The cartridge was washed twice with 2 ml CH_3OH/glacial acetic acid (9:1, v/v) and twice with 1 ml CH_3OH. The cartridge was rinsed dry and elution started slowly with two times 2 ml plus 1 ml CH_3OH/25% NH_4OH (9:1, v/v). The combined eluates were evaporated at 40 C under a stream of nitrogen. The dry residue was reconstituted with 0.5 ml CH_3CN/H_2O (4:6, v/v) using the whirlmix and ultrasonic bath. The suspension then was transferred into 300 μl micro autosampler glass tubes and centrifuged in an Eppendorf centrifuge for three min at 10,000 rpm. The clear supernatant was transferred into another micro autosampler tube and 200 μl injected onto the column.

The modular HPLC system consisted of an ISS-autosampler (Perkin-Elmer), a model 64 HPLC pump (Knauer) and a model 5100 A electrochemical detector (ESA) using 850 millivolts (mV) for the analytical and 900 mV for the guard cell, range 50 × 1. The column (125 × 4.6 mm) was slurry-packed with Nucleosil 5-CN (Machery and Nagel). The flow rate was 1.5 ml/min. The mobile phase consisted of 40% CH_3CN and 60% 0.02 M KH_2PO_4, pH 2.0 (v/v). The retention time for BM 41.448 and BM 41.440 was 9.7 and 8.2 min, respectively. Total analysis time was 15 min. The limit of detection was 15 ng/ml. In order to preserve the necessary sensitivity, the system had to be purged with mobile phase for 30 min under reductive conditions (−850 mV) every morning.Calibration graphs were prepared by assaying samples to which known amounts of BM 41.440 had been added. Peak height ratios of BM 41.440 relative to internal standard were plotted against the known concentrations. The resulting correlations were linear with coefficients of correlation between 0.98 and 0.99.

TABLE 1

Pharmacokinetic Data of BM 41.440 in Rats after a Single Oral Dose of 15 mg/kg bw

Parameter	
C_{max} (mg × l^{-1})	1.7
t_{max} (hr)	7
Cl/f (ml × min^{-1} × kg^{-1})	4.7
AUC, 0–120 hr (mg × hr × l^{-1})	52.9
λ_z	0.026
$t_{1/2}$ (hr)	27.1
C_z (mg × l^{-1})	1.3
A_e, 0–120 hr (5)	1.4

C_{max}, maximum serum concentration; t_{max}, time of maximum serum concentration; Cl/f, ratio of total body clearance/absorption fraction; AUC, area under the curve up to 120 hr; λ_z, rate constant for the terminal elimination phase; $t_{1/2}$, half-life for the terminal elimination, calculated from l_z; C_z, intercept of the extrapolated curve for the terminal elimination with the ordinate; A_e, parent substance excreted in the urine during 120 hr (percentage of administered dose).

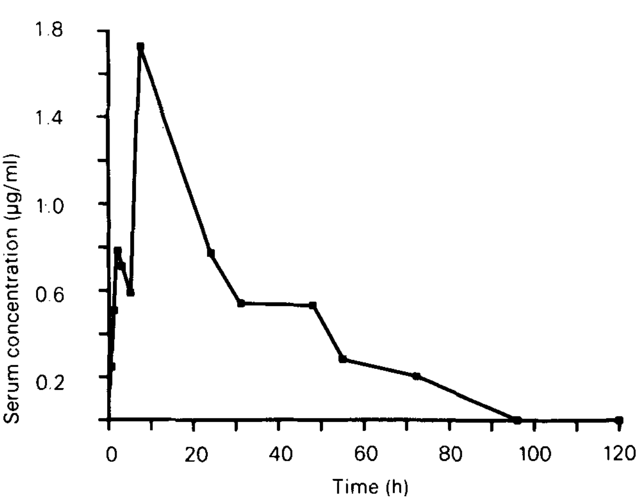

FIG. 1. Mean serum concentration-time profile of BM 41.440 after a single oral dose of 15 mg/kg bw. Blood samples from female rats (n = 4 animals/group, 260–380 g bw) were obtained at the times indicated through permanent catheters placed in the femoral arteries. Extraction of phospholipids and quantification of BM 41.440 was done by means of reverse-phase HPLC.

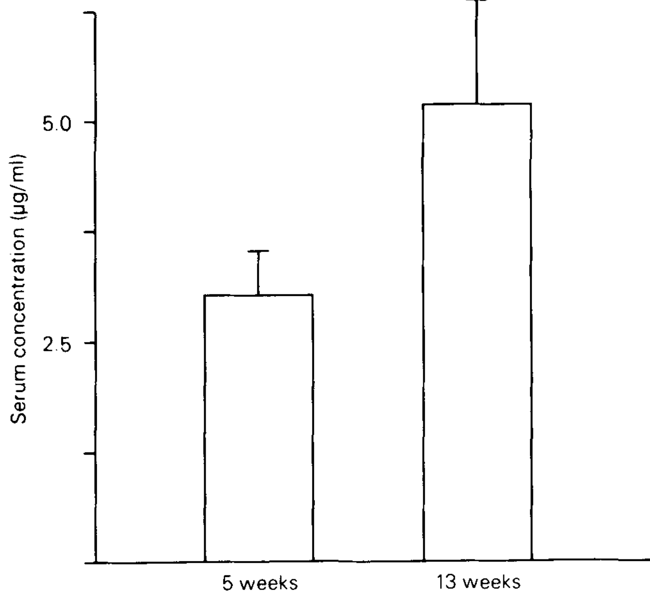

FIG. 2. Mean serum concentrations of BM 41.440 under a daily oral schedule (15 mg/kg bw × day) after five and 13 weeks, respectively. Mean ± S.D., n = 10 animals.

RESULTS AND DISCUSSION

Figure 1 illustrates the BM 41.440 mean serum concentration vs time after a single oral dose of 15 mg/kg bw. Drug concentration increased slowly, and mean peak serum levels (1.7 μg/ml, n = four animals) were attained seven hr after BM 41.440 administration indicating that the thioether analogue is absorbed at a low rate in female rats. A terminal half-life of ca. 27 hr was calculated from the rate constant for the terminal elimination phase ($\lambda_z \sim 0.026$/hr).

All pharmacokinetic parameters are summarized in Table 1. The mean serum BM 41.440 concentration-time-area-under-the-curve (AUC) was 52.9 mg × hr/l. The ratio of total body clearance to absorption fraction was 4.7 ml/min × kg bw.

Recovery of BM 41.440 in urine was examined in all animals receiving a single oral dose of 15 mg/kg bw over 120 hr. In five of 17 animals very small amounts of the drug were found in urine during the first 24 hr. The percentage of administered dose excreted in the urine never exceeded 1.5%. Essentially no drug was detected after 24 hr.

Under a daily oral treatment with 15 mg/kg × day up to 13 weeks, mean BM 41.440 serum concentrations of 3.3 ± 0.5 μg/ml and 5.2 ± 1.2 μg/ml (mean ± S.D., n = 10 animals) were found after five and 13 weeks, respectively (Fig. 2). In accordance with the long half-life and the low excretion rate after single oral administration, these data indicate that accumulation of BM 41.440 can occur in female rats with a calculated factor of accumulation of 2.2 for a dosage interval of 24 hr.

This report is the first pharmacokinetic study done with a non-radiolabeled ether lipid analogue, called BM 41.440 or Ilmofosine. Recently, we described the pharmacokinetic profile of a structurally related alkyl lysophospholipid derivative, 1-[^{3}H]octadecyl-2-methyl-*rac*-glycero-3-phosphocholine, in (Balb/c × C57Bl)F_1 mice (16). This investigation revealed a high in vivo plasma stability of the radiolabeled ether lipid, either after oral or IV administration, with a mean half-life of about 36 hr. So, our results confirmed and extended the findings obtained by others with the same alkyl lysophospholipid analogue in female NMRI mice (17).

Beside avoidance of exposure of humans to radioactivity, the development of a sensitive HPLC technique for determination of ether phospholipids like BM 41.440 offers some major advantages compared to methods utilizing radiolabeled material: (a) in vivo investigations in patients can easily and rapidly be performed in parallel to clinical phase I/II studies, thus providing information on the pharmacokinetics and on the bioavailability of this new class of cytostatics (18) and (b) the compliance of patients entering oral treatment trials can be followed by monitoring the BM 41.440 serum concentrations.

In conclusion, the present data demonstrate that (a) the thioether phospholipid analogue BM 41.440 is absorbed from the gastrointestinal tract after oral administration and (b) under long-term oral treatment high serum concentrations of BM 41.440 can be attained in rats.

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Cytotoxic Activity of the Thioether Phospholipid Analogue BM 41.440 in Primary Human Tumor Cultures

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The inhibitory effect of the new thioether alkyl lysophospholipid analogue 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440, Ilmofosine) on colony formation of different spontaneous human tumors was studied in vitro using a methyl cellulose monolayer assay. The most sensitive tumors were lung (small cell, squamous cell and adenocarcinomas), gastrointestinal and ovarian cancers and hyernephromas. On the basis of the current definition of sensitivity by the National Cancer Institute, Bethesda, MD, i.e. more than 70% inhibition of colony formation at an arbitrary concentration of 10 μg/ml, 34 out of 64 malignancies tested were susceptible to BM 41.440.
Lipids 22, **955–957 (1987).**

Human tumor stem cell assays provide a means of performing drug sensitivity measurements on human tumor cells in primary cultures. Since 1977 when Hamburger and Salmon (1,2) introduced a two-layer soft agar system for cloning tumors taken directly from patients, a number of assays have been developed for a series of human solid cancers to use in selecting the most appropriate chemotherapy for patient's tumor (3–9). Besides the potential use of such colony-forming systems in defining patterns of drug sensitivity, which possibly offers a means of optimizing chemotherapy for individual patients (10), a second potential application of clonogenicity assays is in the area of screening for and developing of new anticancer drugs (11–13).

1-Hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine (BM 41.440, Ilmofosine) (Scheme 1) is one of the most potent cytotoxic alkyllysophospholipids (ALP) (14–16). ALP are analogues of the cell membrane component lysophosphatidylcholine and represent a new class of antitumor agents (17). In a variety of tumor models, these ether lipids have been shown to exert antitumor activity in vitro (15,16,18,19) and in vivo (15,20).

H_2C-O ... R

$HO-CH$

H_2C-OH

SCHEME 1. BM 41.440.

The direct antimalignant effect of ALP is related to a specific interference with the normal phospholipid turnover, preferentially of neoplastic cells (21,22).

This investigation was designed to evaluate the inhibition of human tumor colony formation induced by the thioether analogue BM 41.440, which has recently entered phase I/II trials in human cancer therapy (23).

EXPERIMENTAL

The thioether phospholipid BM 41.440 was supplied as a white, crystalline powder by *Boehringer Mannheim* GmbH (Mannheim, FRG) and stored at 4 C as a stock solution of 100 μg/ml plus 20% FCS.

The culture system used in this study has been extensively described elsewhere (9,24). In brief, single-cell suspensions of spontaneous human tumors to be tested were prepared by mechanical disaggregation. Cell clumps were removed by filtration through sterile nylon sieves down to pore sizes of 20 μm. After repeated washing in serum-free Iscove's modified Dulbecco's medium (IMDM), cells were plated in IMDM plus 30% fetal calf serum (FCS), supported by 0.9% (w/v) methyl cellulose

TABLE 1

Plating Efficiency and Response to BM 41.440 Treatment of Different Spontaneous Human Tumors

Tumor types	Colonies/10^5 cells ± S.D. (range)[a]	Number of sensitive tumors/number of tumors tested[b]
Malignant melanoma	81 ± 30 (41–144)	1/16
Lung		
Small cell	127 ± 51 (41–164)	3/4
Squamous cell	88 ± 11 (80–103)	4/5
Large cell	69 ± 14 (50–83)	1/3
Adenocarcinoma	87 ± 22 (47–108)	4/6
Stomach	90 ± 8 (77–101)	4/5
Colon	76 ± 27 (42–109)	3/5
Ovary	91 ± 36 (50–163)	3/4
Myosarcoma	79 ± 17 (59–101)	3/3
Liposarcoma	65 ± 20 (44–88)	0/3
Hypernephroma	111 ± 16 (84–130)	2/2
Pleuramesothelioma	62 ± 9 (50–72)	2/1
Breast	70 ± 23 (44–90)	1/2
Gall bladder		1/1
Histiocytoma		1/1
Cervix uteri		1/1
Corpus uteri		1/1
		34/64

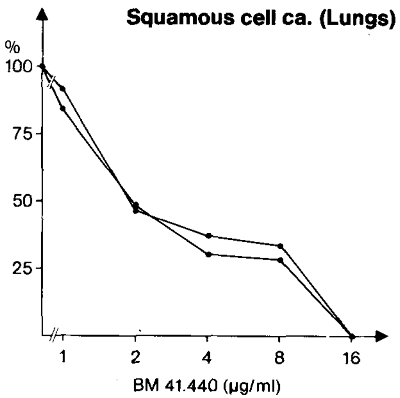
[a]Viable cells (1 × 10^5) were plated (37 C, 7.5% CO_2) in petri dishes in IMDM plus 30% FCS and 0.9% (w/v) methyl cellulose with or without various doses of BM 41.440. After 8–10 days of continuous incubation with the drug, tumor colonies were counted under an inverted microscope. Mean ± S.D., standard deviation.

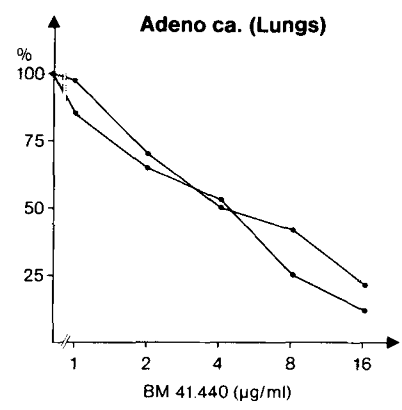
[b]NCI sensitivity criteria: ≥70% inhibition of tumor colony formation at an arbitrary screening concentration of 10 μg BM 41.440/ml.

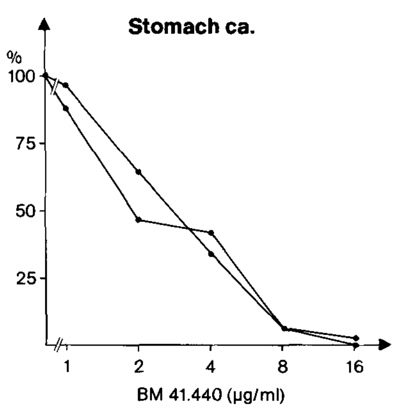
[1]Presented at the symposium on "Ether Lipids in Oncology," Göttingen, Federal Republic of Germany, December 1986.

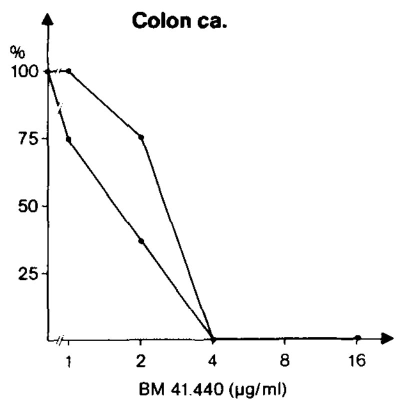
*To whom correspondence should be addressed.

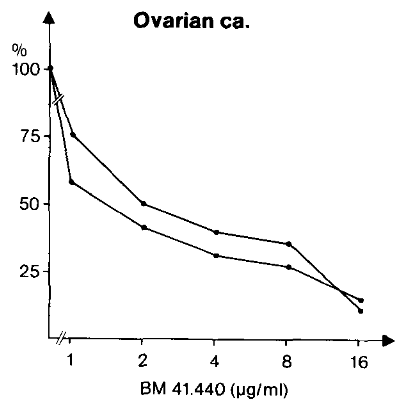
Abbreviations: ALP, alkyl lysophospholipid; BM 41.440, 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine; FCS, fetal calf serum; IMDM, Iscove's modified Dulbecco's medium.











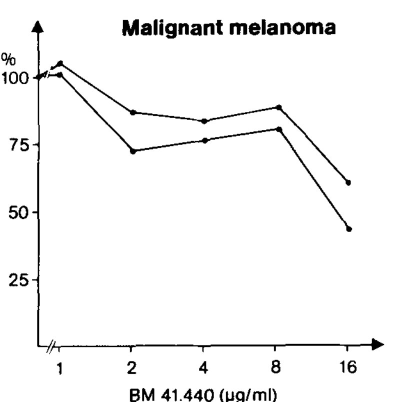


FIG. 1. Dose-response curves of different spontaneous human tumor colonies treated with BM 41.440. Untreated controls are set as 100%. Inhibition is expressed as percent survival of colonies as a function of BM 41.440 concentration. Two specimens of each tumor were tested in duplicates.

and incubated at 37 C, 7.5% CO_2 in a humidified atmosphere with no additional feeding. To assure the presence of an excellent single-cell suspension, positive controls and quality controls, for instance were used as day 1 colony counts and microscopic inspection of control plates were performed immediately after plating. Quality controls have been shown to greatly increase the reproducibility of human colony-forming assays (10). Colonies (>30 cells) usually appeared by days 8–10, and the number of colonies on the plates was determined using an inverted light microscope.

Two specimens of each tumor type were tested in duplicate. Viability of cells was checked by trypan blue exclusion (25). Drug sensitivity of a tumor was defined according to the sensitivity criteria of the National Cancer Institute (Bethesda, MD), i.e. at least 70% inhibition of colony formation at an arbitrary BM 41.440 concentration of 10 μg/ml (10).

RESULTS AND DISCUSSION

The 64 different tumor types evaluated in this study showed a wide range of culturability. Tumor types, plating efficiency and responses to BM 41.440 treatment of the neoplasms tested are listed in Table 1. Approximately 30% of the experiments were judged as unevaluable, mainly due to a lack of adequate growth or to a wide variability of colony counts in control cultures.

The plating efficiency ranged from 0.04 to 0.17%. The data are in accordance with previous findings from our group (9,13,24) and with results reported by others using modifications of the original Hamburger-Salmon technique (3–8,26).

Incubations with BM 41.440 were performed continuously in order to maximize assay sensitivity (27). The in vitro doses of the drug, 0–16 μg/ml, were scheduled empirically according to the concentrations that have been shown to be effective in tumor growth inhibition on different cell lines of murine and human origin (14–17,20).

The tumor colonies showed individual dose-response curves. Some examples are given in Figure 1. The most susceptible malignancies proved to be small cell, squamous cell and adenocarcinomas of the lung, gastrointestinal tumors, ovarian cancers, myosarcomas and hypernephromas with numbers of sensitive tumors (≥70% inhibition of colony formation at an arbitrary BM 41.440 concentration of 10 μg/ml) to numbers of tumors tested of 3/4, 4/5, 4/6, 3/5, 3/3 and 2/2, respectively. Only one out of 16 malignant melanomas and none out of three liposarcomas tested fulfilled the above mentioned sensitivity criteria.

The feasibility and validity of human tumor colony-forming assays for preclinical drug screening and for identifying of, at least, resistance of a tumor to chemotherapy have been shown by several groups (28–33).

Retrospective and prospective clinical trials have revealed true positive rates for colony-forming systems between 47 and 82%, while true negative rates have ranged from 84 to 98% (28–33). Therefore, the present results and the data from the ongoing study on inhibition of tumor colony formation induced by the thioether phospholipid BM 41.440 may provide helpful information for the assessment of indications for BM 41.440 clinical phase II evaluations in the near future.

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In Vitro and In Vivo Cytotoxicity of Alkyl Lysophospholipid ET-18-OCH_3 and Thioether Lipid BM 41.440[1]

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Screening for cytotoxicity in the clonogenic assay in human tumor xenografts and L1210 mouse leukemia revealed comparable dose-dependent effects of the alkyl lysophospholipid ET-18-OCH_3 and the thioether lipid BM 41.440. The efficacy in human tumors only was marginal at low doses. In vivo tests of both agents were carried out in nude mice bearing two of the tumors that proved most sensitive in vitro and in mice inoculated with L1210 leukemia. Only small effects on the growth of the human tumors and no effects on L1210 leukemia were observed. In view of clinical rules for definition of remission, no convincing antitumor effects were obtained.
***Lipids 22,* 958-961 (1987).**

Alkyl lysophospholipids and analogues have gained increasing interest as a new class of potential anticancer agents. Data on activity in various cell lines (1-3), isolated leukemic (2,4) or tumor cells (5,6), and in tumor-bearing animals (1,7), as well as no or minor effects in vitro (3) and in vivo (6,8) have been reported. Furthermore, there are hints that some tumors are susceptible (9,10) and others resistant (9,10) to the same drug under identical conditions. This might indicate that tumor-specific properties cannot be excluded. To gain further insight, additional experimental evidence is required.

Therefore, we tested the alkyl lysophospholipid ET-18-OCH_3 (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) and thioether lipid BM 41.440 (1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine) in a variety of human tumors in the clonogenic assay recommended as the in vitro method for anticancer drug screening (11). The responses of two of the most sensitive tumors in vitro to the tested agents subsequently were studied in nude mice. The combination of both methods especially seems to be promising. It provides the advantage of testing a substance in human material from the beginning under in vitro conditions and allows selection of only the most sensitive tumors for in vivo studies (12). In addition, L1210 mouse leukemia cells were used for experiments in vitro and in vivo since 24 out of 26 clinically used drugs proved active in this model (13) and since immunocompetent mice could be used for therapy trials.

EXPERIMENTAL

Human tumors established in serial passage in NMRI nude mice in our laboratory were used (14-16). L1210 leukemia was obtained from G. Atassi, Brussels, subpassaged and maintained as recommended by the National Cancer Institute (17). Tumors were mechanically disintegrated with scissors and by shear force (Stomacher 80, Colworth, London, UK); they were incubated with enzymes (collagenase 0.04%, DNAse 0.07% and hyaluronidase 0.1%) at 37 C for 30-40 min. Cells were washed and passed through stainless steel sieves of 200- and 50-μm mesh size to obtain single cell suspensions. Cell counts were performed in a hemocytometer, and the percentage of viable cells was determined by trypan blue exclusion. The culture method is a modification of the double-layer soft agar system introduced by Salmon et al. (18) as described recently (12). One ml 0.3% agar (with 30% fetal calf serum and Iscove's modified Dulbecco's medium with L-glutamine) containing 0.8-4.5 $\times$ 10^5 cells/ml (depending on the individual growth rate of each tumor) was pipetted onto 1 ml of 0.5% agar bottom layer (with 10% fetal calf serum) in 35 $\times$ 10 mm culture dishes. Tests were carried out in triplicate.

Drugs were administered in 1 ml medium containing 30% fetal calf serum. Controls received the vehicle only. Additional growth factors were required for the L1210 leukemia (5 $\times$ 10^{-5} mol/l medium 2-mercaptoethanol). Control plates were monitored for growth using an inverted microscope every other day. Final colony counts were obtained with an automatic image analyzer (Omnicon FAS III, Bausch & Lomb, Rochester, New York), at the time of optimal colony formation (5-18 days in culture). In our experience, the optimal time for counting the colonies depended on the individual growth of the tumors, but it was before the medium turned yellow. Colonies had to be ⩾60 μm in diameter and distinct enough so they could be detected separately by the camera. To increase detection, all dishes were incubated with 0.5 ml tetrazoliumchloride (1 mg/ml) 24 hr before the final count. This stains only viable cells and colonies (19). Quality control was achieved by staining plates with tetrazoliumchloride on days 0 and 2 and subsequent freezing at −20 C until the final count was taken after adding 1 ml glycerine.

The criteria for identifying evaluable assays (20) were a slight modification of Shoemaker's approach (11,21). The coefficient of variation in the control group was ⩽50%. A compound was considered active if it reduced colony formation to ⩽30% of the control value. The frozen plates must not exceed ⩾20% counts of the controls. For in vivo testing, two of the most sensitive in vitro tumors were selected, and two tumor slices (size: 5 $\times$ 5 $\times$ 0.5-1 mm) were subcutaneously implanted into the flanks of each of the 4-6 nude mice per group (14,16,22,23). Treatment began when the tumors exhibited an average diameter of 7-8 mm, and the estimated depth was at least half of the smaller diameter. This was reached by the lung cancer on day 24 and by the melanoma on day 31 after transplantation. Animals with yellow tumors reflecting a high amount of fibrous tissue were excluded (24).

Substances were injected intraperitoneally in dosages of 10 and 30 mg/kg body weight (bw) 5-10 times within 12 days. Tumor growth, which was the product of two perpendicular diameters, was recorded every week.

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Abbreviations: ALP, ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; BM 41.440, 1-hexadecylmercapto-2-methoxy-methyl-*rac*-glycero-3-phosphocholine.

Relative tumor size was calculated for each single tumor by dividing its size on day X by its size on day 0 at the time of randomization multiplied by 100. Final evaluation was performed after four weeks. Statistical computation was done by comparing groups with the Mann-Whitney test (25) at the end of the in vivo experiments. This minimized α-inflation. The significance test was done according to Holm (26) at the 5% level.

In vivo experiments with L1210 leukemia were carried out with six CD2F1 mice per drug and dosage and with eight control animals. Cells (10^5) were intraperitoneally (ip) injected and the animals treated with 3, 10 and 30 mg/kg bw ALP or 3 and 30 mg/kg bw BM ip one day later until death. ALP was provided by Andreesen, Medical Department, University of Freiburg, Germany; BM was obtained from Pahlke, Boehringer Mannheim GmbH, Mannheim, Germany.

RESULTS AND DISCUSSION

Table 1 shows the screening results of the clonogenic assay of 22 human tumors. ALP and BM displayed some dose-dependent effects. Efficacy was achieved especially in the lung cancers, whereas the gastrointestinal tumors proved to be more resistant to conforming to their behavior in man. Furthermore, a response of melanomas was demonstrated at low concentrations of ALP and BM. On the whole, growth was suppressed in five of 22 tumors at a concentration of 10 μg/ml. Therefore, this concentration better is suited to select the most responsive tumors with success rates of 61% at 30 μg/ml or 100% at 100 μg/ml. Consequently, the adenocarcinoma of the lung LXFA 526 and the melanoma MEXF 274 were selected for therapy in nude mice.

TABLE 1

Effectiveness of ALP and BM on Human Tumor Xenografts in the Clonogenic Assay

		Concentration (μg/ml)				
Tumor type	Compound	1.0	3.0	10.0	30.0	100.0
Large intestine	ALP		0/1	0/2	0/2	1/1
	BM		0/1	0/2	0/2	1/1
Gastric	ALP		0/1	0/3	1/3	2/2
	BM		0/1	0/3	0/3	2/2
Lung, "small" cell	ALP			1/1	1/1	1/1
	BM			1/1	1/1	1/1
"Large" cell	ALP	0/3	1/4	1/7	2/5	4/4
	BM	0/3	0/4	1/7	3/5	4/4
Melanoma	ALP	0/1	0/2	1/2	1/1	
	BM	0/1	0/2	2/2	1/1	
Sarcoma	ALP			0/2	2/2	2/2
	BM			0/2	2/2	2/2
Mammary	ALP	0/1	0/1	0/1		
	BM	0/1	0/1	0/1		
Thyroid	ALP		0/1	1/1	1/1	
	BM		0/1	1/1	1/1	
Mesothelioma	ALP			1/1	1/1	1/1
	BM			0/1	1/1	1/1
Renal	ALP			0/1	1/1	1/1
	BM			0/1	1/1	1/1
Testicular	ALP			0/1	1/1	1/1
	BM			0/1	1/1	1/1
Human tumors	ALP	0/5	1/10	5/22	11/18	13/13
Total	BM	0/5	0/10	5/22	11/18	13/13
Percent	ALP	0%	10%	23%	61%	100%
	BM	0%	0%	23%	61%	100%

Efficacy: colony number in test ≤30% of control group.

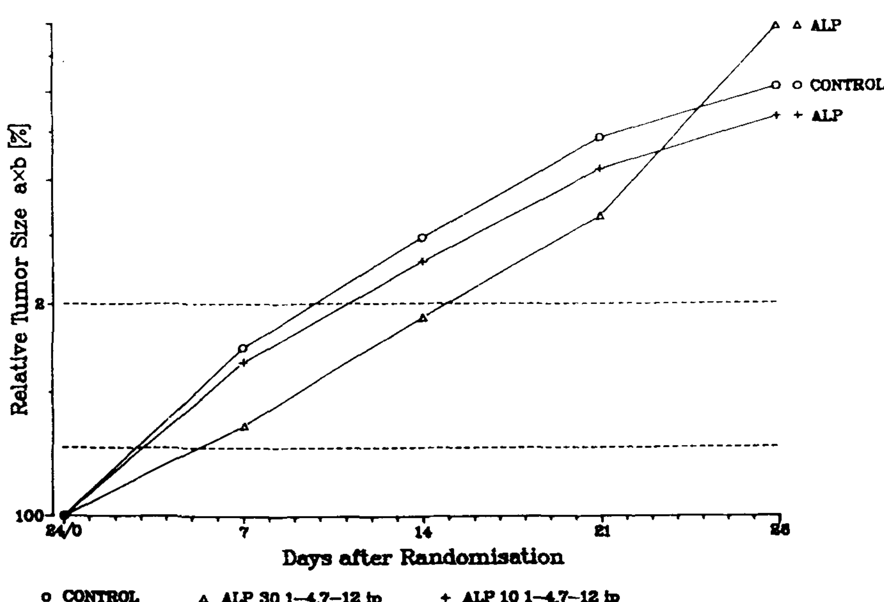

FIG. 1. Relative tumor size during chemotherapy with two doses of ALP (10 or 30 mg/kg bw given days 1-4 and 7-12 ip), number of animals at the beginning/end: control 6/6; 10 mg/kg ALP 5/4; 30 mg/kg ALP 6/4, Holm test at the end of experiment: ns.

Likewise, tests were carried out in mice inoculated with L1210 because this cell line responded to both compounds at 10 μg/ml in our assay. In vivo efficacy of ALP in the lung LXFA 526 is shown in Figure 1. Tumor growth was reduced by ALP at dosages of 10 and 30 mg/kg bw. While this effect was small at the lower dose, it was more apparent at 30 mg/kg bw. However, tumor growth was enhanced as compared with the controls 9–16 days after the last injection. BM was not effective in the same lung carcinoma.

In contrast to the lung cancer, the growth of the melanoma (Fig. 2) was reduced by the therapy with ALP or BM during the first week of treatment; this retardation was not compensated until the end of the experiment. Tests according to Holm revealed growth retardation significance for 30 mg/kg ALP only. Similar, yet more favorable, results are reported for gynecologic tumors (9). Experiments with an anaplastic ovarian carcinoma intravenously or orally treated with ALP especially are comparable. An almost complete growth inhibition was achieved by 500 μg ALP IV twice daily for three weeks. The same dose given orally or only half of it given by IV resulted in a slower proliferation compared with controls. Some differences between these and our experiments should not be overlooked. Two of the 10 mice in each IV-injected group experienced a complete remission (9). However, these beneficial effects were not obtained for two other gynecologic tumors treated identically (9). Certainly, there are differences between the above-mentioned and our procedure concerning the application schedules and the onset of therapy. Compared to others, we administered drugs for a short time, imitating common clinical strategy since the promising approach of long-term application yielded inconsistent results (8,10). The time until the initiation of therapy may be essential for its outcome because our method avoids the phase of ischemic regression after the tumor transplantation (24).

In view of the different results with the various tumors, it seems most likely that tumor-specific properties are extremely significant for success or failure of therapy trials. This assumption is in accord with Berger and Schmähl (27), who like others (8) could not observe any effects of ALP or BM in acetoxymethyl methylnitrosamine-induced rat colonic adenocarcinomas, whereas BM displayed activity superior to ALP in methylnitrosourea-induced rat mammary carcinoma.

With regard to the known effects of clinically introduced cytostatic agents in the L1210 in vivo (13), our results with ALP and BM may be of some interest (Table 2).

Both substances did not increase the survival time of the inoculated animals. This was surprising for us because, first, since like others (28) we found the ip cell and drug application to be very sensitive to drugs and superior to ip cell and IV drug injection (29). Second, both compounds possessed cytotoxic activity in vitro. Third, application into the same compartment appeared ideal for direct cytotoxic mechanisms. Furthermore, ip application of ALP increases the amount of peritoneal macrophages, which seem to mediate ALP activity (31). It appears that L1210 is not very susceptible to alkyl lysophospholipids, which is consistent with observations made in other laboratories (30; Munder and Vogler, personal communication).

In conclusion, ALP and BM exhibited only limited antitumor activity in vivo in two of in vitro's most sensitive malignancies. If one attempts to imitate "clinical conditions," i.e., if one uses human material, high tumor burden, tumors adapted to their hosts and discontinuous treatment, no convincing antitumor effects are observed. Especially, remissions are not achieved. However, a comparison with the literature suggests that some tumors may be more sensitive to ALP and BM. Although the final significance of all currently reported data is difficult to judge, it is encouraged to design further studies that concentrate on the questions of tumor specificity and optimized applications.

Chemotherapy of MEXF 274

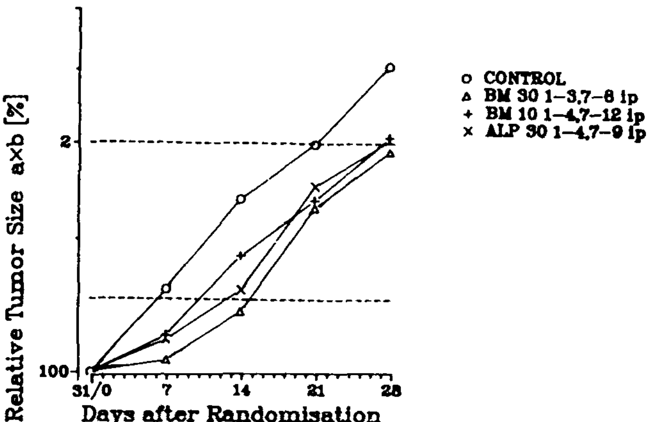

FIG. 2. Relative tumor size during chemotherapy with one dose ALP and two doses BM (30 mg/kg ALP given days 1–4 and 7–9; 10 mg/kg BM days 1–4, 7–12; 30 mg/kg BM 1–3, 7–8) ip, number of animals at the beginning/end: control 4/4, 30 mg/kg ALP 5/4; 10 mg/kg BM 5/5; 30 mg/kg BM 5/3), Holm test at the end of experiment: $P_{ALP} = 0.014$, $\alpha^* = 0.017$; for BM ns.

TABLE 2

Effectiveness of ALP and BM on the L1210 Mouse Leukemia

	Dosage (mg/kg/day)	Lifespan median (days)	Lifespan range (days	Cures/total
ALP	30	7	6–9	0/6
	10	7.5	2–9	0/6
	3	8	8	0/6
BM	30	8.5	7–9	0/6
	3	8	7–8	0/6
Controls		8	7–9	0/8

10^5 cells ip into CD2F1 ♀ and ♂ mice. Therapy: day 1–9, ip, dissolved in 0.9% NaCl.

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Phase I Trial of the Thioether Phospholipid Analogue BM 41.440 in Cancer Patients[1]

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BM 41.440 (1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine) is a new thioether phospholipid, which has been shown to possess antineoplastic, antimetastatic, anti-invasive and immunomodulating properties in several tumor models. The mechanism whereby this compound exerts its direct antineoplastic effect is thought to be related to specific interference with the normal phospholipid metabolism, preferentially of neoplastic cells. BM 41.440 was evaluated in a multicenter phase I study in patients (pts) with refractory cancers. In phase I A, 34 pts were orally treated with doses ranging from 0.5 to 7.0 mg/kg body weight (bw). Three different formulations were tested. The maximum-tolerated dose (MTD) was ca. 5 mg/kg bw. The limiting side effects were nausea and vomiting. There was no evidence for systemic toxicities like myelosuppression, nephro-, neuro-, hepatotoxicity or hematological side effects. The current phase I B is designed to determine the MTD of BM 41.440 administered orally on a daily schedule for at least eight weeks. So far, 19 pts have entered this trial at dose levels ranging from 1.0 to 5.0 mg/kg bw/day. Some pts receiving 1.0 and 2.5 mg/kg bw/day, respectively, have been treated, up to now, for more than nine months. Clinical progress was followed with at-least-weekly blood counts, chemistry profiles, urine analysis, liver function tests and recordings of side effects. Tumor parameters were evaluated at eight-week intervals. In parallel, pharmacokinetic investigations were performed in some pts in phase I A and IB. First results on tolerability and therapeutic efficacy of the long-term BM 41.440 treatment are reported in this intermediate evaluation.
Lipids 22, 962–966 (1987).

BM 41.440 or 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine (prop. INN: Ilmofosine) is a synthetic 1-S-thioether alkyllysophospholipid derivative (1) (Fig. 1). Alkyl lysophospholipids (ALP) are structurally related to lysophosphatidylcholine, which is a minor constituent of most cellular membranes (2,3) and plays an important role as a metabolic intermediate in the continuous turnover of the fatty acid moiety of phosphatidylcholine via the deacylation/reacylation cycle (Lands pathway) (4,5). ALP represent a new class of cytostatic drugs (6). The antitumor activity has been studied in different tumor models in vitro (7–9) and in vivo (6,10). The mechanism of direct tumor cell destruction by BM 41.440 and other ALP is related to a selective interference of these compounds with the cellular phospholipid metabolism of tumor target cells (9,11–13).

$$
\begin{array}{l}
\qquad\qquad\quad H_2C-S-C_{16}H_{33} \\
\qquad\qquad\quad\ | \\
H_3C-O-CH_2-CH \qquad O \\
\qquad\qquad\quad\ | \qquad\quad\ \ \| \\
\qquad\qquad\quad H_2C-O-P-O-(CH_2)_2-\overset{\oplus}{N}(CH_3)_3 \\
\qquad\qquad\qquad\qquad\quad\ | \\
\qquad\qquad\qquad\qquad\quad O^{\ominus}
\end{array}
$$

FIG. 1. Chemical structure of BM 41.440.

BM 41.440 was selected for clinical trials because preclinical screens demonstrated differential activity on malignant cell types of murine and human origin (1,14,15). Moreover, notable antitumor effects within in vivo tumor systems were observed, including the methylcholanthrene-induced fibrosarcoma (MethA), L1210 leukemia, Lewis lung carcinoma (1,14) and the methylnitrosourea (MNU)-induced mammary carcinoma (16). BM 41.440 caused significant reduction in tumor size and significant increases in the life span of mice bearing these tumors. Antitumor activity in the MNU-induced mammary carcinoma was dose-dependent. Recent data also indicate that BM 41.440 has antineoplastic activity against different spontaneous human tumor cells in a methyl cellulose colony-forming system (17,18).

Animal toxicology studies conducted in rats and dogs showed the rats to be the more sensitive species (14). The side effects of oral BM 41.440 were non-hematological and included vomiting and diarrhea.

Here, we report the interim results of the first clinical phase I study of BM 41.440 in adult patients with refractory cancers. We also include a preliminary examination of drug pharmacokinetics.

EXPERIMENTAL

Drug formulation. BM 41.440 was synthesized by Boehringer Mannheim GmbH, 6800 Mannheim, Federal Republic of Germany. In this study, three different formulations were tested: a soft gelatine capsule, a film tablet and a coated, gastric juice–resistant film tablet. The drug was supplied in 25, 50 and 100 mg quantities. Quality controls for all formulations were performed before submission for application in patients according to international standards.

Patient selection. The present clinical phase I trial of BM 41.440 was divided into two parts: part I A and I B. Patient characteristics are shown in Table 1 and Table 2. Up to now, a total of 53 patients with histologically

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Abbreviations: ALP, alkyl lysophospholipid; BM 41.440, 1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine.

TABLE 1

Patient Characteristics—Phase I A

Characteristics	Measurement
No. of patients in study	34
No. of eligible/evaluable patients	34
Men	20
Women	14
Mean age (years)	59 (43–70)[a]
Mean Karnofsky performance status (%)	90 (60–100)
Tumor types	
Adenocarcinoma (lung)	12
Squamous cell (lung)	2
Large cell (lung)	1
Small cell (lung)	1
Malignant melanoma	8
Adenocarcinoma (colon)	7
Hypernephroma	2
Squamous cell (oesophagus)	1

[a]Number in parentheses, range.

TABLE 2

Patient Characteristics—Phase I B

Characteristics	Measurement
No. of patients in study	19
No. of eligible/evaluable patients	17
Men	10
Women	9
Mean age (years)	56 (33–68)[a]
Mean Karnofsky performance status (%)	90 (80–100)
No. of patients with prior treatment	15
Surgery	4
Chemotherapy	2
Chemotherapy + radiotherapy	1
Surgery + chemotherapy	5
Surgery + radiotherapy	2
Surgery + chemotherapy + radiotherapy	1
No. of patients with no prior treatment	4
Tumor types	
Non-small cell (lung)	4
Small cell (lung)	1
Malignant melanoma	7
Adenocarcinoma (colon)	3
Pancreatic gland carcinoma	1
Parotid gland carcinoma	1
Hypernephroma	1
Unknown primary	1

[a]Number in parentheses, range.

documented, advanced malignancies refractory to known forms of effective therapy were entered into this trial. Thirty-four patients were entered into phase I A and 19 patients into phase I B. Other eligibility criteria included patients ranging from 18–70 years old, an ambulantory performance score of ≥50% (Karnofsky), no anticancer therapy for at least four weeks before entering the study, life expectancy of at least three months, and adequate organ function as defined by bone marrow (white blood cell count ≥4000/mm³, platelet count ≥100,000/mm³), liver (bilirubin ≤1.5 mg/dl, normal SGOT, SGPT, γGT and AP values) and kidneys (creatinine ≤1.5 mg/dl). Informed consent was obtained from all patients before therapy began.

Ineligibility criteria were malabsorption and/or maldigestion, pregnancy, psychotic disorders, metastases in the brain or brain tumors, major organ disfunctions, e.g. cardiac insuffficiency, and active systemic infections.

Study design. The study protocol was reviewed and approved by the ethical boards of the universities of Freiburg, Munich and Mannheim. The study was performed following German regulations. The primary objectives in phase I A were (a) determination of the maximum-tolerated dose (MTD) of a single or multiple oral administration of BM 41.440 for one day, (b) characterization of possible toxic effects and (c) definition of the dose-limiting toxicities according to the World Health Organization grading (19). A minimum of two patients evaluable for toxicity were treated sequentially at each dose level, starting with a low dose. The dose levels in phase I A were 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg/kg body weight (bw). After the second patient was treated with a given dose and evaluated for toxicity, patients were entered at the next higher dose level. The dose was escalated until a dose-limiting toxicity was observed.

The objectives of the phase I B were (a) determination of maximum-tolerated dose and of possible side effects of a continuous daily oral administration over at least eight weeks and (b) determination of the responses to BM 41.440 treatment according to the WHO criteria for "Reporting Results of Cancer Treatment" (19). In this part of the study, a minimum of four patients were treated at each dose level. The starting dose was 1.0 mg/kg bw/day. Dose escalations proceeded from 1.0 to 2.5 to 5.0 mg/kg bw/day. If after four weeks unacceptable toxicity did not occur at a specific dose level, the next dose level was started. There was no escalation of dose for any individual patient.

Patient monitoring. Prior to therapy, all patients underwent a complete history and physical examination. Patients who were entered into the long-term treatment of phase I B were comprehensively evaluated on measurable diseases by the appropriate modality, including a physical examination, x ray, scans and other studies as necessary for documentation of the extent of the diseases. Pretreatment evaluation included complete blood count with white blood cell (WBC) count differential, hemogram, full chemistry profile, creatinine, calcium, uric acid, blood sugar, liver function tests, lactate dehydrogenase, cholesterol, protein, prothrombin, thrombin time and urine analysis. In phase I A, patients were carefully monitored on day 1, 2 and 7 for toxicity by repeated physical examinations, complete blood counts with WBC differential, chemistry profiles and all other parameters listed above. During the time of treatment in phase I B, patients were followed closely for signs of toxicity and for all study parameters at a minimum of weekly intervals up to eight weeks. At that time, an evaluation of tumor size was done by physical examination, appropriate radiological studies and scans, and by tumor markers. WHO standard tumor response criteria were used to evaluate the antitumor effect of the drug (19). These criteria included (a) complete response (disappearance of all known disease, determined

TABLE 3

Tolerability of BM 41.440 (Phase I A)

Dose (mg/kg × day)	No. of patients with side effects/ no. of patients treated	Nausea (WHO grade)[a]			Diarrhea (WHO grade)[b]
		1	2	3	1
0.5	0/2	0	0	0	0
0.75	0/2	0	0	0	0
1.0	0/2	0	0	0	0
2.0	1/3	1	0	0	0
3.0	2/6	0	1	2	1
3.0 (Sg. capsule)[c]	2/4	0	1	2	1
3.0 (Tablet)	0/2	0	0	0	0
4.0	2/3	1	1	0	1
5.0	2/3	0	2	0	0
6.0	3/4	0	2	1	1
7.0	7/7	3	2	1	6
7.0 (Tablet)	3/3	1	1	1	2
7.0 (Fc. tablet)[c]	4/4	2	1	0	4

Patients were treated orally with single or multiple (up to four times) doses of BM 41.440 for one day. Three different formulations were tested: soft gelatine capsule, film tablet and coated film tablet. The no. of patients with side effects to no. of patients treated and the side effects are separately listed for each formulation administered for the particular dose levels 3.0 and 7.0 mg/kg bw, respectively, on the two lines under the appropriate dosage.

[a]WHO grade 1, nausea only; grade 2, transient vomiting; grade 3, vomiting requiring therapy.

[b]WHO grade 1, transient (≤2 days); grade 2, tolerable but >2 days.

[c]Formulations: Sg. capsule, soft gelatine capsule; Fc. tablet, film-coated tablet resistant to gastric juice.

by two observations not less than four weeks apart); (b) partial response (50% or more decrease in total tumor size of the lesions, which have been measured to determine the effect of therapy by two observations not less than four weeks apart), (c) no change (a 50% decrease in total tumor size cannot be established nor has a 25% increase in the size of one or more measurable lesions been demonstrated) and (d) progressive disease (25% or more increase in the size of one or more measurable lesions, or the appearance of new lesions).

If patients showed tumor response or showed no changes after eight weeks of treatment with BM 41.440, the therapy was continued as long as no progression occurred. In these cases, patients were monitored with study parameters every second week up to 12 weeks of treatment and at a minimum of four-week intervals thereafter. Assessments of the disease's extent to determine antitumor activity of the drug were performed at eight-week intervals. Individual patients were removed from the study if they experienced unacceptable toxicity or if objective disease progression occurred.

Pharmacokinetics. Sixteen out of 34 patients in phase I A were studied following the single oral BM 41.440 administration. Blood samples were obtained immediately before starting with the drug application and then 0.5, 1, 2, 4, 6, 8, 12, 24 and 32 hr as well as seven days after p.o. doses. Blood samples were centrifuged, and the plasma was decanted and stored frozen at −20 C until analysis. BM 41.440 was measured by a newly developed high-pressure liquid chromatography method, using a structurally closely related analogue of BM 41.440 as an internal standard. Details on the analytical technique for detection of BM 41.440 are published in this issue (20).

RESULTS

Tolerability. Fifty-three patients were entered on this phase I study; 34 patients were treated on the one-day schedule in phase I A. The patients' characteristics are shown in Table 1. Of the 34 patients entered on phase I A, all were evaluable for toxicity. Drug-related effects were nonhematological. The number of patients treated at each dose level and the toxicities are summarized in Table 3. Gastrointestinal irritations were seen in 17 out of the 34 patients entered on this part of the trial. The dose-limiting toxicities were nausea and vomiting, WHO grades 1–3, and diarrhea, WHO grade 1.

Two out of four patients treated with the soft gelatine capsule at a dose level of 3.0 mg/kg bw showed gastrointestinal side effects (Table 3). However, neither of the two patients treated with the coated film tablet experienced similar toxicity at the same dose level. Therefore, dose escalations from 3.0 to 7.0 mg/kg bw were continued with the coated film tablet formulation. At the dose level of 7.0 mg/kg bw, three out of three patients showed gastrointestinal intolerabilities. Therefore, the same dose was administered to four other patients using a coated film tablet that is resistant to gastric juice.

TABLE 4

Tolerability of BM 41.440 (Phase I B)

Dose (mg/kg × day)	No. of patients with side effects/ no. of patients treated	Nausea (WHO grade)[a]			Diarrhea (WHO grade)[b]	
		1	2	3	1	2
1.0	1/4	1	0	0	0	0
2.5	5/6	3	1	0	1	1
5.0	5/7	1	2	2	4	0

Patients were daily treated p.o. with single or multiple (up to three times) doses of BM 41.440 for at least eight weeks (film tablet).

[a,b]For WHO-grading, see legend to Table 3.

However, these four patients all also experienced dose-limiting toxicities, indicating that changing the formulation did not benefit the patients.

Up to now, 19 patients were in the current long-term treatment study of phase I B. The patients' characteristics are shown in Table 2. Fifteen of these patients were pretreated by different therapy modalities; four patients had not received any cancer treatment prior to BM 41.440 therapy. Seventeen out of the 19 patients entered on this part of the study were evaluable for toxicity. The two inevaluable patients had just entered the study when the compilation of this intermediate report was performed.

As seen in phase I A, dose-limiting toxicity consisted of gastrointestinal irritations. Eleven out of 17 patients developed nausea and vomiting, WHO grades 1-3, and diarrhea, WHO grades 1-2 (Table 4).

No other toxicities were observed, neither in phase I A nor in phase I B. Although some patients were treated on a daily oral schedule with BM 41.440 for more than 36 weeks, up to now there was no evidence for myelosuppression, hepatic or renal toxicities, allergic reactions, hypotension or hematological side effects.

The maximum-tolerated dose of BM 41.440 administered as a single or multiple oral dose for one day in adult patients was around 5 mg/kg bw (Phase I A). For a long-term treatment with BM 41.440 on a daily oral schedule, doses up to 5.0 mg/kg bw/day can be recommended for treatment of more than nine months with the film tablet.

Clinical responses. The current clinical phase I B trial of oral BM 41.440 in 12 patients with refractory cancers who were evaluable for antitumor activity revealed three partial responses. In one patient with a metastatic adenocarcinoma of unknown primary localization, two liver metastases (2 cm × 2 cm, 3 cm × 3 cm) completely disappeared after eight and 15 weeks of treatment, respectively. In another patient with a metastatic malignant melanoma, a more than 50% reduction of two liver metastases (9 cm × 9 cm, 5 cm × 5 cm) occurred within eight weeks of treatment with BM 41.440. However, in both of these patients, the disease progressed after 26 and 10 weeks, respectively. In a third patient with a malignant melanoma, one liver metastasis (3 cm × 3 cm) disappeared within eight weeks of therapy. Another lung metastasis remained constant for, up to now, 36 weeks. These three patients have been treated with 1.0 mg BM 41.440/kg bw/day.

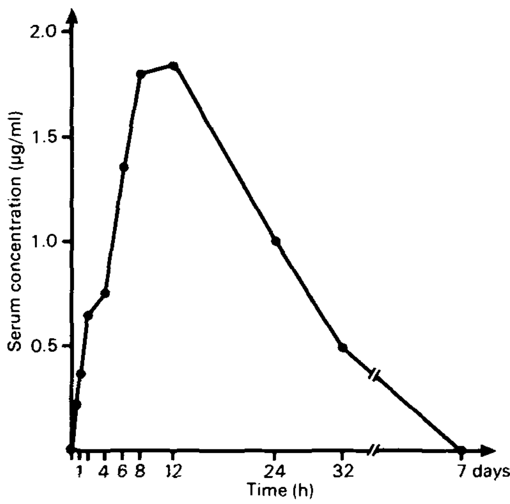

FIG. 2. Time course of plasma BM 41.440 (1-hexadecylmercapto-2-methoxymethyl-*rac*-glycero-3-phosphocholine) concentrations after a single oral administration to a patient with an adenocarcinoma of the lung. Dose: 4 mg/kg bw (275 mg).

Furthermore, out of 12 patients evaluable for response, six patients had no observable changes with durations, up to now, of seven to 24 weeks (three adenocarcinomas of the lung, two malignant melanomas, one metastatic parotid gland carcinoma). Progressive disease was seen in three other patients. Four patients dropped out of the study, three of them due to side effects.

Pharmacokinetics. Figure 2 illustrates a BM 41.440 plasma concentration vs time profile for a patient with an adenocarcinoma of the lung, treated with 4 mg/kg bw (275 mg) BM 41.440 p.o. (film tablet). Mean peak plasma concentrations of 1.8 µg/ml were attained within 8-12 hr after BM 41.440 administration. In phase I B, pharmacokinetic parameters were studied in all patients who had been entered into the trial. The summary of the results of the pharmacokinetic investigations will be given in the final report.

DISCUSSION

BM 41.440 is an interesting compound because of its completely different mechanism of tumor cell destruction as compared to all known cytostatic drugs (9,11-13,15). Its antineoplastic, antimetastatic and anticarcinogenic activity has been investigated in different in vitro and in vivo tumor models (14-16,18,20-22). In this current multi-institutional phase I trial, BM 41.440 was administered orally on a one-day and daily schedule of at least eight weeks, respectively. The dose-limiting toxicities in both parts of the study were nausea, vomiting and diarrhea. Hematological or other non-hematological side effects related to BM 41.440 treatment were essentially absent until now. In phase I A (one-day administration), drug formulation was changed at a dose level of 3.0 mg/kg bw. Since the soft gelatine capsule seemed to be less tolerable, further dose escalations were continued using a BM 41.440 film tablet up to 7.0 mg/kg bw. Changing drug formulation to a coated film tablet, which is resistant to gastric juice, at a dose level of 7.0 mg/kg bw did not enhance tolerability.

Similar results in side effects were obtained from our preliminary experience on the long-term treatment schedule (phase I B). Doses up to 5.0 mg/kg given daily for at least nine months seem to be quite safe. In this part of the study, the toxicities observed, e.g. nausea, vomiting and diarrhea, appear to be dose-related (Table 4).

The preliminary pharmacokinetic data in this phase I study revealed gastrointestinal absorption of BM 41.440 after single oral administration. Further explorations that are aimed at clarification of the metabolism and the major routes of elimination of BM 41.440 in humans are underway.

Although three clinical responses and six no changes were observed in the long-term treatment part of this trial, the limited number of patients entered, tumor types studied, and extent and heterogeneity of prior therapy preclude conclusions about the antitumor efficacy of this drug. However, these intermediate results encourage us to introduce BM 41.440 into clinical phase II studies.

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Clinical Phase I Pilot Study of the Alkyl Lysophospholipid Derivative ET-18-OCH_3[1]

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Sixteen patients suffering from widespread malignant disease, the majority pretreated and found in poor general health, were treated in a phase I pilot study with the alkyl lysophospholipid derivative 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3). Eleven patients were treated intravenously, and five were given oral therapy. Prolonged IV administration of 15-20 mg/kg/day at a concentration of 5 mg ET-18-OCH_3 per 1 ml 20% human serum albumin could be continued safely. The maximum-tolerated dose was either 50 mg/kg as a single injection or 20 mg/kg during daily dispensions. Grade 2-4 toxicity, as pulmonary edema and impairment of hepatic function, then occurred during daily treatment. Toxicity was reversible. Mitogen stimulation and mixed lymphocyte culture studies revealed possible immunosuppressive effects of higher doses of ET-18-OCH_3. There were no chromosomal changes in cytogenetic studies. Frequent post-mortem examinations revealed no further toxicity. IV and oral treatment showed few encouraging response data since there were two partial remissions in non-small cell lung cancers and a reduction of leukemic blasts to less than 10% in an acute myelomonocytic leukemia.
Lipids 22, **967-969 (1987).**

Alkyl lysophospholipid (ALP) derivatives represent the first generation of ether lipids with antineoplastic properties in vitro and in vivo. A detailed review of this area of investigation has been published recently (1). Some of these compounds have substantial therapeutic efficacy in various rodent tumor systems. This activity has been interpreted to be mediated partially by cytotoxic effector cell populations, e.g. macrophages and direct effects of this material on tumor cells as cytotoxicity, induction of differentiation and anti invasiveness. After toxicologic investigations (1), which included studies with subhuman primates (Munder and Andreesen, personal communication), we have introduced 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) into a first clinical phase I pilot trial (2,3).

MATERIALS AND METHODS

Sixteen patients with solid tumors (seven non-small cell lung cancers, one bowel carcinoma, one hypernephroid carcinoma, one urothelial carcinoma, one adrenal cortex carcinoma, one thyroid gland carcinoma, one glioblastoma) and leukemias (one acute myelomonocytic leukemia, two chronic myeloid leukemias/blast crisis) were treated in our institution from 1979-1982. There were 11 male and five female patients. The median age of those with solid tumors was 64 years (range: 19-85 years). The median age of the leukemic patients was 37 years (range: 17-39 years). With the exception of two patients with non-small cell lung cancers, all patients have been refractory to prior therapy as surgery, radiation or chemotherapy; ten patients had obtained multi-modality treatment before entering the study. All patients suffered from metastatic or widespread disease, and the performance score (Karnofsky index) before the study was below 50 for the majority of the patients. Written informed consent of the patients was obligatory.

The chemical structure of the ALP 1-*O*-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine (ET-18-OCH_3) is given in Figure 1. The compound was synthesized according to methods modified from procedures previously published (4-6), stored in a desiccator at −20 C, dissolved aseptically in 20% human serum albumin (HSA) up to a concentration of 5 mg/ml and infused intravenously at 37 C. For oral treatment, the ET-18-OCH_3 was dissolved in milk (3.5% fat content) at various concentrations. Routine checks on purity (chromatography), sterility (microbiology) and pyrogen content (animal experiments) were performed on each batch.

The study was performed in accordance with German law. The study protocol has been reviewed and approved by an ethical board. Eleven patients were treated intravenously. For dose finding, we began with single applications of 2 mg/kg, which is 10% of the lowest dose that produces moderate toxicity in toxicologic studies with subhuman primates (Munder and Andreesen, personal communication). Single application doses then were increased by doubling the amount of ET-18-OCH_3 and beyond 10 mg/kg by adding smaller amounts. Some patients were treated by prolonged daily applications, beginning with 1 mg/kg/day and using the same dose escalation procedure. Five patients were treated orally, following a similar dose escalation but starting with daily applications. Since we faced a shortage of the noncommercial drug during this first pilot study, dose increases for individual patients were performed at due intervals to obtain a maximum of information.

Prior to treatment, the following studies were performed on every patient: a case history, A (chest x ray,

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H2C—O—(CH2)17—CH3
 |
HC—O—CH3
 |          O
 |          ||                  ⊕
H2C— O—P—O—(CH2)2—N(CH3)3
            |
            O⊖
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FIG. 1. Chemical structure of ET-18-OCH_3.

[1]Presented at the symposium on "Ether Lipids in Oncology," Göttingen, Federal Republic of Germany, December 1986.

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electrocardiography, echocardiography, abdominal sonography, pulmonary function, tumor/leukemia staging with the necessary examinations, Coombs-tests, bone marrow) and B (physical examination, performance-status, weight, cellular blood count including erythrocyte morphology, reticulocytes, erythrocyte osmotic resistance, differential white blood count, thrombelastography, blood sedimentation rate, serum [blood urea nitrogen, creatinin, uric acid, electrolytes, bilirubin, glytamic oxalo-acetic transaminase, glytamic pyruvic transaminase, γ-glutamyl transferase, alkaline phosphatase, cholinesterase, gamma-lactate dehydrogenase, lactate dehydrogenase, amylase, lipase, creatinin phosphokinase, β_2-microglobulin, haptoglobin, hemopexin, plasmatic coagulation, triglycerides, cholesterin, glucose, electrophoresis, immunoglobulins, complement C_3 and C_4 and antinuclear factors], creatinin-hippurane-clearance, urine-profile, urine-protein, urine-glucose and urine-electrolyes). A-values were repeated when necessary, at least once every month and at the end of the treatment. B-values were repeated at least once every week. Other examinations, such as thyroid hormones and chromosome analysis in peripheral blood cells, were done facultatively.

Toxicity was determined according to the Southeastern Cancer Study Group criteria with some minor modifications. Maximum-tolerated dose (MTD) was defined as the occurrence of grade 2 (moderate) toxicity after single or during daily applications and was evaluated for IV treatment.

For response evaluation partial remission (PR) has been defined as regression of all measurable tumor parameters of more than 50% with a remission duration for at least one month. Tumor regression of less than 50% for more than one month has been referred to as minor response (MR). Progressive disease (PD) was stated if one or more tumor parameters or white blood count in patients with leukemia developed progressively under therapy, paralleling a drop in performance status. No change (NC) or complete remissions (CR) have not been observed.

RESULTS AND DISCUSSION

Eleven patients were treated intravenously. Generally, toxicity was mild, i.e. grade 1 of the Southeastern group criteria. Two patients developed transient indurative erythema, which so far lacks a pathophysiological explanation on some parts of their skin. Six patients experienced gastrointestinal side effects of vomiting and diarrhea, which we found to be independent of dose level but correlated with the concentration of ET-18-OCH_3 in HSA and the infusion rate. Both side effects could be avoided by choosing an ET-18-OCH_3 concentration in 20% HSA, not exceeding 5 mg/ml, and by keeping the infusion rate below 20 ml per hr. Grade 1 liver toxicity was observed in three patients as an impairment of hepatic synthesis function monitored by a temporary drop in serum cholinesterase, and in one occasion as a drop of the Quick test. One patient showed grade 2 liver toxicity, as there was a cholestasis with increased alkaline phosphatase and bilirubin. Grade 1 renal toxicity was found in two patients with an elevation of serum creatinine and glucosuria, and proteinuria was found in one case. Life-threatening toxicity (grade 4) was observed in one patient, who developed an interstitial pulmonary edema, which correlated with a change of performance status. Similar pulmonary edema in a second patient reached only grade 2 toxicity because of prompt drug removal. Grade 1–2 toxicity was found to be reversible within a few days after removal of ET-18-OCH_3; it did not require any special treatment. Grade 4 pulmonary edema was found to be completely reversible under assisted ventilation and corticosteroids with the drug removed.

The MTD during single IV applications, given to five patients with weekly intervals, was 50 mg/kg infused within 24 hr. This dosage was reached in two patients, and the limiting factor was the infusion rate, since gastrointestinal toxicity occurred. MTD during daily applications was 20 mg/kg/day, reached by four patients. One patient could be treated with 20 mg/kg/day for 10 days without grade 2 toxicity. The other patients experienced either liver or lung toxicity reaching grade 2 or more (see Table 1). Additionally, after accidental extravasal infusion, there were two episodes of thrombophlebitis.

Five patients were treated orally. Grade 1 toxicity was concentrated on the gastrointestinal tract, hepatic function and renal function, and it had the same characteristics as those induced by IV treatment. Five mg/kg/day could be given safely to four of the patients for longer time intervals, 10 mg/kg/day produced life-threatening pulmonary edema after 10 days in one patient, which was completely reversible under short-term assisted ventilation and corticosteroids with the drug removed. However, oral MTD was not established in this pilot trial since better galenic formulations of this type of drug are under way.

Mitogen stimulation and mixed lymphocyte culture (MLC) studies in the blood lymphocytes of six patients were performed to reveal possible in vivo immunosuppression of higher doses of ET-18-OCH_3, which had been found in vitro (7). The results so far remain contradictory. However, since there was a significant decrease of mitogen stimulation and MLC reactivity in one patient, which showed reversibility after the end of the treatment, further studies in this direction should be performed.

Cytogenetic studies have been performed in lymphocytes of patients under treatment with negative results (8). Pathological and histological post-mortem examinations were performed in eight of the 16 patients who died of progressive disease after various time intervals. There were no further signs of toxic alterations exceeding the common findings in advanced neoplastic disease and death.

TABLE 1

Maximum-Tolerated Dose (MTD) and Limiting Toxicity for IV ET-18-OCH_3

- MTD for single infusion of ET-18-OCH_3 (20% HSA, 5 mg/ml): 50 mg/kg

  Limiting toxicity: Gastrointestinal toxicity (nausea, diarrhea)

- MTD for daily infusions of ET-18-OCH_3 (20% HSA, 5 mg/ml): 20 mg/kg/day

  Limiting toxicity: Lung, liver

Details of response and survival data are in previous reports (2,3,9). In summary, there were two PR in intravenously treated patients with non-small cell lung cancer, which were controlled by both x ray and bronchoscopy, and showed a remission duration for five and six months. Minor responses were found in one patient with a hypernephroid carcinoma and another patient with a thyroid gland carcinoma. In a patient with an acute myelomonocytic leukemia that was treated intravenously, we observed a ca. 1-log reduction of leukemic blasts in the peripheral blood ($78.7 \times 10^3/\mu l$ to $3.4 \times 10^3/\mu l$) within 15 days of treatment with a parallel recovery of granulopoiesis. However, this did not qualify for response since we could not obtain a second bone marrow examination from this patient. All other patients were found with progressive disease. However, all patients finally died from progressive disease; none of the patients survived longer than one year after study.

In summary, we have treated 16 patients suffering from widespread malignant disease; the majority has been refractory to previous treatments and was found to be in poor general performance. MTD for single IV administration of the drug was ca. 50 mg/kg with nausea and vomiting as the limiting toxicity. Prolonged daily IV applications of doses less than 20 mg/kg/day at 5 mg ET-18-OCH_3/1 ml 20% HSA seemed to be safe. Limiting toxicity occurred in liver and as interstitial pulmonary edema giving 20 mg/kg/day. The etiology of the pulmonary edema remains speculative. However, residual platelet-aggregating effects of the ET-18-OCH_3 similar to the chemically related platelet-activating factor (PAF) may play a role (10). Thus, it seems to be essential to look for antitumor ether lipids and derivatives for future clinical trials without PAF effects. On the other hand, typical side effects for antitumor cytotoxics as myelosuppression or alopecia were not found within the study; the drug also remained genetically inactive.

The purpose of this study performed with a limited amount of drug was only to obtain basic information on the applicability and possible value for further clinical testing of this material in cancer patients. Since we have partially characterized the tolerability of this first antineoplastic ether lipid have found some limited antitumor activity of the material, the information obtained from this pilot trial represents basis and justification for further clinical testing of related structures within a wider scope of phase I and II trials (see also Herrmann, D. et al., this issue).

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REVIEW

Ether Lipids and Analogs in Experimental Cancer Therapy. A Brief Review of the Munich Experience[1]

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This review covers the work of our laboratory on the antineoplastic activity of some ether lipids and derivatives that are related to platelet-activating factor (PAF). Various 1-*O*-alkyl lysophospholipid derivatives (ALP) show therapeutic activity in mouse transplant tumor models and in metastatic 3-Lewis lung carcinoma in vivo. However, certain autochthonous mouse leukemias and radiation-induced lymphomas are resistant to ALP treatment. The therapeutic effects of these compounds are partially due to the activation of cytotoxic macrophages and direct cytotoxicity.

Approximately 20 ether lipids and derivatives were tested for direct cytotoxicity in cells from human solid tumors and leukemias using [^{3}H]thymidine uptake, trypan blue dye exclusion, human tumor clonogenic assays (HTCA) and cell morphology as criteria. Certain ALP, thioether lysophospholipid-derivatives (TLP), ether-linked lipoidal amines, *sn*-2 analogs of PAF, and conjugates of ether lipids and cytosine arabinoside were found cytotoxic in a dose- and time-dependent fashion. Cytotoxicity of some of the ether lipids tested is based on destruction of cell membranes. Structure-activity studies were performed to better understand the mechanisms leading to accumulation and cytotoxicity of ALP. Comparative studies with normal bone marrow cells and leukemic blasts from humans revealed preferential antileukemic cytotoxity of three ether lipids.
***Lipids 22,* 970–973 (1987).**

In the early seventies, three independent laboratories observed antitumor effects of certain ether lipids for the first time. Munder et al. (1) examined synthetic analogs of 2-lysophosphatidylcholine (2-LPC) and reported prophylactic and therapeutic efficacy of certain alkyl lysophospholipid (ALP) derivatives in some allogeneic and syngeneic mouse transplantation tumors. These ALP originally were synthesized as a new class of biological response modifiers. Figure 1 depicts the chemical structures of some first generation ALP. Ando et al. (2) reported on therapeutic effects of certain alpha-glycerylethers in the Ehrlich ascites tumor. Boeryd et al. (3) described therapeutic activity of methoxy-substituted alkyl-glycerols in some metastasizing sarcomas induced by methyl-cholantrene in mice.

Whereas the alpha-glyceryl ethers and alkyl-glycerols so far have not led to the systematic development of anticancer drugs that are applicable in clinical oncology, this attempt is under way with different ALP and other ether lipid-derivatives. This review briefly summarizes the contribution of our laboratory in this area.

[1]Presented at the symposium on "Ether Lipids in Oncology," Göttingen, Federal Republic of Germany, December 1986.

$H_2C-O-(CH_2)_{17}-CH_3$ / $HC-O-CH_3$ / $H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

ET-18-OCH$_3$

$H_2C-O-(CH_2)_{17}-CH_3$ / HCH / $H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

ET-18-H

$H_2C-O-(CH_2)_{17}-CH_3$ / $HC-OH$ / $H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

ET-18-OH

$H_2C-O-(CH_2)_{11}-CH_3$ / HCH / $H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

ET-12-H

$H_2C-O-C(=O)-(CH_2)_{16}-CH_3$ / $HC-OH$ / $H_2C-O-P(=O)(O^{\ominus})-O-(CH_2)_2-N^{\oplus}(CH_3)_3$

2-LPC

FIG. 1. Chemical structures of some representative alkyl lysophospholipid-derivatives (ALP) of the first generation and of the ester-lysophospholipid 2-LPC.

RESULTS OF ANIMAL EXPERIMENTS

When it became evident that ALP could inhibit the growth of different primary tumors in mice, we began studying the influence of these compounds on experimental metastasis, using the 3-Lewis lung carcinoma (3-LL) growing in syngeneic $C_{57}Bl_6$-mice (4,5). In a first series of experiments, we observed only minor activity of the ALP, trying to retard the growth of 3-LL primary tumor. However, application of the compounds either shortly before or shortly after the surgical removal of the primary tumor significantly retarded and inhibited the 3-LL metastasis; this was demonstrated by an increase of the median survival time and the number of surviving animals. In these experiments, the compounds showed

activity when they were given intradermally, intravenously or even orally. Among various ALP, the analogs with a long aliphatic side chain in the *sn*-1 position of the molecule and a substitution of the *sn*-2 position of the molecule, e.g. with a methyl-group, showed the highest activity.

Subsequently, in vitro and in vivo experiments performed with macrophages from the peritoneal cavity or the bone marrow of $C_{57}Bl_6$ mice could show that the inhibition of metastasis of 3-LL by ALP partially is mediated by an activation of macrophages to a cytotoxic effector cell population after contact with these compounds. In these experiments, macrophages from the peritoneal cavity of $C_{57}Bl_6$ mice could be rendered cytostatic and cytotoxic for 3-LL cells in vitro by a preincubation with the lipids. Furthermore, pure syngeneic bone marrow macrophages have shown some therapeutic antimetastatic activity in vivo when used in the 3-LL. However, preincubation of these macrophages with various ALP in vitro yielded in a significantly improved therapeutic antimetastatic activity in vivo when these cells then were intravenously reinjected into the animals after surgical removal of the primary tumor, and the incidence of death from metastasis was observed.

In the experiments summarized above, the ALP had to be given daily for a minimum of three weeks to produce visible therapeutic effects. In contrast, with some of our new conjugates of ALP or TLP with 1-β-D-arabinofuranosylcytosine (Ara-C) a treatment duration of only five days was necessary to inhibit the intraperitoneal growth of the 3-LL primary tumor or to inhibit the development of metastasis of the 3-LL with an adjuvant treatment approach. Figure 2 shows four of the lipid-ARA-C conjugates, including the most active Ara-CDP-D,L-PBA and Ara-CDP-D,L-PTBA. (Further details concerning these experiments are published in a separate paper of this *Lipids* issue.)

In another series of experiments, we so far could not show prophylactic efficacy of the ALP ET-18-OCH_3 or ET-18-OH on the development of radiation-induced lymphomas in syngeneic $C_{57}Bl_6$-mice or the spontaneous development of leukemias in AKR-mice (6). The reason for the lack of therapeutic activity of the lipids in these tumor models remains unknown. However, experiments with radiation-induced mouse tumors, which use a wider dose range of the lipids, are underway.

RESULTS OF HUMAN TUMORS AND LEUKEMIAS EXPERIMENTS IN VITRO

More recently, we performed another series of experiments to study the phenomena of macrophage activation and direct cytotoxicity of certain ether-lipid derivatives with human tumors and leukemias in vitro. Over 30 lipids and lipid-derivatives were tested for cytotoxicity in more than 100 human tumors and leukemias. We used the tritiated thymidine uptake, the trypan blue dye exclusion, the human tumor clonogenicity assay (HTCA), and microscopic and scanning electron microscopic (SEM) procedures as test systems. In these experiments, 1-*O*-alkyl-lysophospholipid-derivatives (ALP), thioether-lysophospholipid-derivatives (TLP), ether-linked lipoidal amines, analogs of the platelet-activating factor (PAF), PAF-antagonists and conjugates of lipids and cytotoxics revealed cytostatic and cytotoxic activity in neoplastic cells of human origin.

Figures 1, 2 and 3 show representative structures of these lipids and lipid-derivatives. In general, more than 24 hr of incubation were necessary to obtain full cytotoxic efficacy of the compounds. Besides this relation to time, there was a clear dose-response relationship for the cytotoxic activity of the vast majority of the compounds; There were few exceptions. ET-18-analogs of ALP (see

ara-CDP-DL-PCA

ara-CDP-DL-PBA

ara-CDP-DL-MBA

ara-CDP-DL-PTBA

FIG. 2. Chemical structures of four lipid conjugates of 1-β-D-arabinofuranosylcytosine (in cooperation with C. I. Hong, Roswell Park Memorial Institute, Department of Neurosurgery, Buffalo, NY).

FIG. 3. Representative chemical structures of four other groups of ether-lipid-derivatives. A: BM 41.440. A thioether-lysophospholipid derivative (TLP) (Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany)' B: 1-*O*-alkyl-2-acetamide-*sn*-glycero-3-phosphocholine. A PAF-analog (J. Hajdu, Department of Chemistry, California State University, Northridge, CA); C: CP-46.665. A lipoidal amine (Pfizer Central Research, Groton, CT); D: SRI 63-154. A PAF-antagonist (Sandoz Research Institute, East-Hanover, NJ).

TABLE 1

Cytotoxicity of ET-18-analogs (ET-18-OCH$_3$ and ET-18-H) of 2-LPC in Tumors and Leukemias of Human Origin In Vitro

	Number of tumors/leukemias	
Type of disease	Sensitive	Resistant
Prostate carcinomas	2	
Testicular seminoma	1	
Teratocarcinomas	3	
Bladder carcinomas	2	
Hypernephroid carcinomas	17	2
Astrocytomas	2	
Glioblastomas	2	
Meningioma	1	
Medulloblastoma		1
Carcinoma of the ovary	1	
SCLC[a]	1	
NSCLC	1	
NHL/B	1	
NHL/T	1	
AMML	4	
AML	3	
ALL	2	
HL-60	1	
K-562		1
CML/BC	6	
Total	51	4

[a]SCLC, small-cell lung cancer; NSCLC, non-small cell lung cancer; NHL/B (T), non-Hodgkin lymphoma of B (T)-cell origin; AMML, acute myelomonocytic leukemia; AML, acute myeloid leukemia; ALL, acute lymphocytic leukemia; CML/BC, chronic myeloid leukemia/blast crisis.

Fig. 1), which were extensively examined in this respect, have shown a clear dose-response relationship between the concentrations of 1–10 μg/ml. The majority of tumors and leukemias tested were sensitive to the cytotoxic properties of, e.g., the ET-18-analogs of ALP, and they showed viability of less than 50% when incubated with concentrations of these compounds <20 μg/ml for at least 48 hr. However, it is important that there also were certain cell types that proved resistant to this material. Examples are given in Table 1. For the group of ALP, an alkyl-ether-linkage in the *sn*-1 position of the molecule and a substitution of the *sn*-2 position of the molecule were necessary for reproducible cytotoxicity. The ester-lysophospholipid 2-LPC, which always was tested in parallel as a reference compound for direct detergent-like surface activity, has shown either none or minor activity within the same dose range. The lyso-PAF ET-18-OH was cytotoxic in only some tumors and leukemias, furthermore, this cytotoxicity could be inactivated by preincubation with a microsomal tetrahydropteridin-requiring 1-*O*-alkyl-cleavage enzyme. Different laboratories have shown that this enzyme is present in a variety of normal tissues but characteristically absent from neoplastic cells (for further literature, see ref. 7). Further structure-activity analysis with the D- and L-forms of the ET-18-OH in the HL 60 leukemic cell line showed that the activity of the racemic compound is due to the activity of the D-form (8). However, this could not be reproduced by testing various freshly explanted human leukemic cell samples including patients refractory to, or with relapses after previous chemotherapy (for details, see the publication of Danhauser et al., this issue of *Lipids*). Furthermore, microscopic and SEM studies could show that the cytotoxicity of ALP, TLP, the lipoidal amine CP-46,665

and the PAF-analogs is correlated with destruction of the outer cell membranes. Detailed results of our investigation concerning the cytotoxic properties of these lipids and lipid derivatives have been published before (for further literature, see ref. 7).

In an additional series of experiments, we were able to show that preincubation of monocytes/macrophages of human origin with certain ether lipids in vitro renders these cells cytostatic for autologous tumor cells of the same patient (9). This effect was observed with various human hypernephroid carcinomas and non-small cell lung cancers but not with human brain tumors. Cytotoxity studies with six ether lipid derivatives comparing the sensitivity of bone marrow to leukemia cells revealed preferential antileukemic cytotoxity of three lipids, including ET-18-OCH_3 and BM 41.440 (for further details, see the publication of Schick et al., this issue of *Lipids*).

DISCUSSION

There is increasing experimental evidence that certain ether lipids and derivatives represent a new group of antineoplastic compounds. This notion is supported by the results of first clinical pilot trials (10). The activity of these structures partially is mediated by tumor cytotoxic cells and due to direct effects of this material on neoplastic cells. These direct effects consist of cytostatic-cytotoxic properties discussed here and also consist of the induction of the differentiation in neoplastic cells (11) and an inhibition of the invasive properties of neoplastic cells (12). Although the molecular mechanisms leading to these direct effects are yet unknown, accumulation of these lipids in neoplastic cells, disturbance of lipid metabolism, and destruction of cell membranes seem to be important. ET-18-OCH_3 and other ALP represent a first generation of toxic ether lipids that are now widely used as reference compounds. The high synthetic potential in lipid chemistry possibly will open a new area for the acquisition of toxic molecules useful for experimental anticancer therapy. Although a deeper understanding of the molecular mechanisms of this cytotoxicity might be helpful in drug design, the value of these structures for clinical oncology has to be shown in the various in vitro and in vivo activity-screening systems and finally proven in clinical trials.

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Concluding Remarks

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Synthetic ether lipids and related substances, originally conceived as analogs of natural lysolecithin, have been established as a new class of tumoricidal agents, first by Munder about 10 years ago and confirmed by many others. Ether lipids produce strong biological signals!

Organic chemists have synthesized series of analogs in which the glycerol substituents of natural lysolecithin have been varied in several directions, especially by Eibl and his group, and also by others. I would also like to mention, by the way, the elgant stereospecific PAF-synthesis by Mangold.

Biochemists have done much work on the biosynthesis and metabolism of ether lipids, concentrating, for example, on the 1-*O*-alkyl cleavage enzyme system elaborated by Snyder. Enzymic studies included the phospholipases A_2, C and D and other hydrolases, as well as acyltransferases, their occurrence and relative activity in normal and malignant cells and, especially, in cell plasma membranes and in the endoplasmic reticulum.

Natural ether lipids have been known for some time, but interest was greatly stimulated by the findings of Hanahan and others that the platelet-activating factor (PAF) is a 1-*O*-alkyl glycerol derivative. Also, work on plasmalogens and on their metabolism should be mentioned as well as observed genetic aberrations in ether lipid metabolism that occur in Zellweger syndrome, as described by van den Bosch.

Data were presented on the influence of alkyl lysophospholipids (ALP), such as ET_{18}-OCH_3, on phospholipid biosynthesis, turnover and degradation. Of special interest appears to be the inhibition by ALP of phosphorylation reactions, for example, those catalyzed by protein kinase C.

Membranologists have contributed to the understanding of the relation of the physicochemical make-up of ether lipids with regard to their influence on membrane dynamics, such as the change in membrane properties with regard to uptake and/or release of biologically active materials, hormones, etc., by synthetic ether lipids, including the cholesterol ethers discussed by Stein. Changes that occur in membrane-bound enzyme reactions is another demonstration of the potential importance of the new substances, as are findings about the binding of ether lipids and their metabolites to cellular subfractions.

Many interesting remarks were made on new and more sophisticated types of liposomes for various clinical applications (reduced toxicity and/or prolonged action of drugs, liposome-bound antigens plus adjuvants, etc.) or about liposomes as vehicles with affinity to specific organs, such as the liver.

Biologists and clinical investigators have studied the action of ether lipids on tumor and normal cells in vitro and in vivo. Several promising substances have tumor prophylactic as well as inhibitory or even, hitherto in animals, curative properties. Some representatives of the new class seem to act selectively enough on certain, but not all, tumor cells that further intensified work appears to be warranted. Some model types of ether lipids have been studied extensively. The development of possibly still more efficient antitumor compounds can now be based on biochemical, pharmacological and biomedical criteria at hand. This means that coming from phenomenology, we have gone a good way towards a more logical approach. We are at a beginning in an exciting field; our optimism appears justified.

Reports on the first clinical studies, notably on far-advanced cancer cases, seem to call for further efforts and have emphasized the importance of combined basic and applied clinical research. We are still discussing possible mechanisms of the tumoricidal action of ether lipids and its selective character. Candidates are macrophage- (and probably also NK cell-) activation, immune tumor cytotoxicity, direct toxic effects on malignant cells with disturbance of their membrane lipid metabolism, enzyme inhibition or activation, etc. Some clinicians, like Andreesen, stressed the importance of studying mediators, such as interleukin I and II, under ALP treatment. Also, anti-infective and radio-protective properties of the substances were stressed.

There are good reasons not to restrict our attention only to the antitumor activities of ether lipids but also to consider, for example, suppressive effects on certain hitherto not well-controlled autoimmune conditions.

It should be kept in mind that, apparently, one and the same ether lipid is not equally effective on all kinds of tumors. In comparative studies, some tumors are more, others are less, sensitive some may be even resistent. As an example, we may remember the selective cytotoxicity of Eibl's hexadecyl phosphocholine on mammary tumors, as Schmähl and Berger stated, or the alkylglycerol "cascade" of Unger and Eibl in the treatment of skin tumors. Most of the synthetic ALP tested so far were racemic. However, Inoue observed a higher biological activity of the "natural" L-isomer, *sn*-1, of ET_{18}-OCH_3 on macrophages and a somewhat higher toxicity of the D-isomer in vivo but not in vitro.

The antiinvasive properties of ALP were beautifully demonstrated in mixed cell cultures by Storme. There were also contributions showing changes in the sialic acid conjugation or incorporation by ALP into cell surface glycosyl groups, which seem to influence invasive properties of cells.

Of great interest is the relatively high sensitivity of leukemic cells towards ALP and the induction of differentiation of immature stem cells by low ALP doses. One of the possible applications was discussed by Vogler: by purging of bone marrow stem cell cultures taken from leukemic patients, with ALP the leukemic cells can be almost or fully eliminated without damaging normal cells, so that auto-retransfusion of the "purified" cultures could be envisioned.

Thus, there seem to be various possibilities for clinical applications of ether lipids, which should be carefully followed.

We were bombarded with so much interesting and new data that we will go home enriched with many ideas for further work in our field of interest and expertise. Publication of our discussions in the journal *Lipids* will help to keep our memory of these days awake. Personally, I feel that we should try to keep contact and to further exchange information as much as possible.

LETTER TO THE EDITOR

A Suggested Shorthand Nomenclature for the Eicosanoids

Sir:

In preparing comprehensive reviews of the eicosanoid literature for the CRC handbook of eicosanoids (1,2), it was necessary to summarize the known eicosanoids biosynthesized from arachidonic acid and related polyenoic precursors. In doing this, we had to standardize some useful nonambiguous shorthand nomenclature for oxygenated fatty acids (HETEs, for example) that did not form part of the prostaglandin, leukotriene or other well-named families of eicosanoids. As minimal criteria, we wanted the nomenclature system to (1) reflect previous usage as much as possible; (2) avoid ambiguity; and (3) be easy to use in spoken communication.

There currently is some confusion regarding the use of a shorthand nomenclature for hydroxyeicosapolyenoic acids. Previously in the literature, some discrepancies have existed among authors in devising their own shorthand versions using a "HETE"-type nomenclature. For instance, some authors have named dihydroxy derivatives of arachidonic acid "diHETEs," while others have used "DHETEs." With the discovery of epoxide-containing eicosanoids, this has been even more confusing. For example, 10-hydroxy-11(12)-epoxy-5-*cis*,8-*cis*,14-*cis*-eicosatrienoic acid has been called "EPHETA" by one group (3) and "10,11,12-HEPA" by another (4). In addition, some related compounds have been named (hepoxilins, trioxilins, for example) by Pace-Asciak and colleagues (5).

For newcomers to the field, systematic nomenclature is described in great detail in the eicosanoid handbook (1,2) but we present here a summary of this overview with emphasis on an abbreviated nomenclature.

As a beginning framework, all of the universally accepted and used shorthand nomenclature systems also are used in this system. These include the classic prostaglandins, which have nomenclature based upon the prostanoic acid system first introduced by the Karolinska group (6); thromboxanes A_2, B_2, A_3, B_3 (as TXA_2, etc.), which is used with a systematic nomenclature that may be based either on the original heptadecenoic acid system (7,8) or on the more recent thrombanoic acid system (9); and the leukotriene nomenclature proposed by Samuelsson's group (10,11). In addition, the more recently proposed names "trioxilins" and "hepoxilins" by Pace-Asciak (5) and "lipoxins" ("LX") for recently described compounds by Serhan and Samuelsson (12,13) also are used here, since these compounds may have important and unique biological effects. Examples of some other compounds for which shorthand names may be derived

FIG. 1. Conversion of arachidonic acid to 12-HETE; hepoxilin A_3 (8,11(12)-HEpETrE); trioxilin A_3 (8,11,12-TriHETrE); hepoxilin B_3 (10,11(12)-HEpETrE); and trioxilin B_3 (10,11,12-TriHETrE) (1,5).

FIG. 2. Conversion of eicosapentaenoic acid to 15-HpEPE, to 5,15-DiHpEPE, and to lipoxene A (5,6,15-TriHEPE) and lipoxene B (5,14,15-TriHEPE) via postulated 5(6),15-EpHEPE intermediate (1,23).

from common or original usage include Bc-PGE_2 (bicyclo PGE_2) (14); LGD_2 and LGE_2 (levuglandins D_2 and E_2) (15); and $PnGA_3$ and $PnGA_4$ (punaglandins 3 and 4) (16), with "A" designation added because of a PGA-ring in their structures. (See Reference 1.)

For the other eicosanoids, we have adopted the following rules: (1) Oxygenated substituents on the fatty acid chain. (a) These are numbered in sequence from the usual terminal (number 1) carboxyl group of the fatty acid molecule. Wherever an epoxide (or oxane bridge) occurs, the position of the carbon atom covalently linked also is defined but in parentheses: for instance, the 5(6)-epoxide derived from arachidonic acid by a cytochrome P-450 dependent monooxygenase system (17,18). (b) To describe the nature of these substituents, we have adapted slightly and standardized the common usage of other authors. For instance, "H" denotes hydroxy; "Hp" denotes hydroperoxy; and "Ep" denotes epoxy. The use of the lower case letter "p" denoting peroxy is done to avoid a confusion with the large "P," which is used in this nomenclature to denote the five double bond (pentaenoic) system. (c) To describe the number of hydroxyl or other substituents, we suggest standardizing the use of "Di" for 2, "Tri" for 3, "Tetra" for 4, etc. (e.g.: 5,15-DiHETE). For monohydroxy, no such prefix is required and previous common usage is retained. Use of "TH" (as in THETE, etc.) would present ambiguity because it could mean *either* "tri" or "tetra"; and use of "DH" or "TrH" would produce names that would be difficult to vocalize. (2) Number of double bonds and chain length. After description of the nature and position of substituents, the number of double bonds and chain length is described by adaptation of the usual nomenclature. Thus, "ETE" means eicosatetraenoic acid ("T" always denoting tetra and the final "E" denoting enoic acid). By contrast, "ETrE" means eicosatrienoic acid. Again, the lower case is used to avoid the ambiguity previously existing in this case between "trienoic" and "tetraenoic." For example, the heptadecatrienoic acid formed as a byproduct of the cyclooxygenase enzyme once commonly was called "HHT." We propose that it now should be called 12-HHTrE and HHTE now would designate a tetraenoic acid derivative. Notation of chain length is based on previous common usage, unless severe ambiguity results. The "H" denoting heptadeca (C_{17}); "O" denoting octadeca (C_{18}); "N" denoting nonadeca (C_{19}); and "T" denoting tetradeca (C_{14}) are adopted from previous long-term common usage (e.g., the old "HHT," now 12-HHTrE). By contrast, we reserve use of "HD" for hexadeca (C_{16}), which avoids confusion with heptadeca. This is consistent with our suggested use of "DD" for dodeca (C_{12}), which avoids confusion with "D" for docosa (C_{22}). Using these criteria, 13-hydroxyoctadecadienoic acid (from linoleic acid) (19) would then be called (as previously) 13-HODE; and the shorthand form for 8 and 9 carbon compounds, if such were found later, could be "octa" and "nona."

Descriptive examples of this nomenclature are shown in Table 1. Examples of structures of some of these compounds derived from arachidonic acid and eicosapentaenoic acid are shown in Figures 1 and 2.

Are there any ambiguities remaining in this system for shorthand nomenclature of the eicosanoids? It could be argued that "H" could mean either hydroxy or heptadeca. However, this is dealt with by designation of position number and order of appearance. For example, in 12-HHTrE, the first "H" (immediately following position number) means hydroxy, whereas the second "H" (appearing after the hydroxy substituent), means heptadeca.

Another possible ambiguity is the position of the double bonds. This was not a disadvantage to the original Holman system (20) (described below) since the fatty acids had no substituents and all-*cis* methylene interrupted double bonds (skipped unsaturation) always were assumed. However, in the case of substituted fatty acids the original position of the double bonds is often altered, and the original all-*cis* configuration might change in different ways. This drawback can be minimized by defining use of the shorthand (at least once in any paper using it) in terms of structure or systematic name, provided for all known eicosanoids in the review by Smith in *Handbook of the Eicosanoids* (1). Alternatively, we suggest using the Δ nomenclature within parentheses to indicate position of double bonds. For example, 5,15-DiHETE could be called ($\Delta^{6,8,11,13}$)5,15-DiHETE. Should *trans* configuration occur, as in this example, further elaboration (at the expense of brevity) also is possible by denoting

TABLE 1

Examples of Shorthand Nomenclature

Precursor	Compound	Position number prefix	Substituent prefix		Chain length		Suffix: Number of unsaturations		Comments
			Symbol	Description	Symbol	Description[a]	Symbol	Description	
Arachidonic acid (20:4ω6)	5-HETE	5-	H	Hydroxy	E	Eicosa (20)	TE	Tetraenoic acid	
	12-HHTrE	12-	H	Hydroxy	H	Heptadeca (17)	TrE	Trienoic acid	Cyclo-oxygenase by-product
	15-HpETE	15-	Hp	Hydroperoxy	E	Eicosa (20)	TE	Tetraenoic acid	
	5(6)-EpETrE	5(6)-	Ep	Epoxy	E	Eicosa (20)	TrE	Trienoic acid	
	12(S)-HETE	12(S)-	H	Hydroxy	E	Eicosa (20)	TE	Tetraenoic acid	(S) and (R) denote sterochemistry of hydroxy group (see ref. 2)
	12(R)-HETE	12(R)-	H	Hydroxy	E	Eicosa (20)	TE	Tetraenoic acid	
	5,15-DiHETE	5,15-	DiH	Dihdroxy	E	Eicosa (20)	TE	Tetraenoic acid	
	13,14(15)-HEpETrE	13,14(15)-	HEp	Hydroxyepoxy [13] [14(15)]	E	Eicosa (20)	TrE	Trienoic acid	
	5(6),15-EpHETE	5(6),15-	EpH	Epoxyhydroxy [5(6)] [15]	E	Eicosa (20)	TE	Tetraenoic acid	
	14-Oxo-TTE	14-	Oxo-	Oxo (aldehyde)	T	Tetradeca (14)	TE	Tetraenoic acid	
	12-Oxo-DDTrE	12-	Oxo-	Oxo (aldehyde)	DD	Dodeca (12)	TrE	Trienoic acid	
Dihomo-γ-linolenic acid (20:3ω6)	12-HETrE	12-	H	Hydroxy	E	Eicosa (20)	TrE	Trienoic acid	
	8,9,12-TriHEDE	8,9,12-	TriH	Trihydroxy	E	Eicosa (20)	DE	Dienoic acid	
	8,15-DiHpETrE	8,15-	DiHp	Dihydroperoxy	E	Eicosa (20	TrE	Trienoic acid	
	12-HHDE	12-	H	Hydroxy	H	Heptadeca (17)	DE	Dienoic acid	Cyclo-oxygenase by-product
	10,11(12)-HEpHE	10,11(12)-	HEp	Hydroxyepoxy [10] [11(12)]	H	Heptadeca (17)	E	(mono)enoic acid	Possible cyclo-oxygenase product
Adrenic acid (22:4ω6)	14-HDTE	14-	H	Hydroxy	D	Docosa (22)	TE	Tetraenoic acid	
	14-HNTrE	14-	H	Hydroxy	N	Nonadeca (19)	TrE	Trienoic acid	Cyclo-oxygenase by-product
Eicosapentaenoic acid (20:5ω3)	12-HEPE	12-	H	Hydroxy	E	Eicosa (20)	PE	Pentaenoic acid	
	5,15-DiHpEPE	5,15-	DiHp	Dihydroperoxy	E	Eicosa (20)	PE	Pentaenoic acid	
	12-HHTE	12-	H	Hydroxy	H	Heptadeca (17)	TE	Tetraenoic acid	Cyclo-oxygenase by-product
Mead acid (20:3ω9)	(5,8,10t)12-HpETrE (previously [ref. 1] ω9-12-HpETrE)	(5,8,10t)12- (previously [ref. 1] ω9-12-)	Hp	Hydroperoxy	E	Eicosa (20)	TrE	Trienoic acid	ω9 (Holman nomenclature [ref. 20]) distinguishes this from 12-HpETrE formed by 12-oxygenation of 20:3ω6
Docosahexaenoic acid (22:6ω3)	14-HpDHE	14-	Hp	Hydroperoxy	D	Docosa (22)	HE	Hexaenoic acid	
	10,13,14-TriHDPE	10,13,14-	TriH	Trihydroxy	D	Docosa (22)	PE	Pentaenoic acid	
Linoleic acid (18:2ω6)	13-HODE	13-	H	Hydroxy	O	Octadeca (18)	DE	Dienoic acid	

[a]Number of carbons is in parentheses.

stereo chemistry of the double bonds as c *(cis)* or t *(trans)*, hence ($\Delta^{6t,8c,11c,13t}$)5,15-DiHETE. However, we suggest that an easier shorthand for *routine* use would be to put the double bond position in parentheses, without the Δ symbol, and only insert t (for *trans*) when needed. For example, 13-HODE could be elaborated as (9,11t)13-HODE (Table 1).

We feel that there now is a pressing need for a system of shorthand nomenclature of the eicosanoids that (like ours) accommodates previous usage and does not artificially force people into an officially designated system that may be clumsy and not related to previous common usage. For example, Holman (20) popularized the use of ω-6, ω-3 to define nomenclature of the polyunsaturated fatty acids, and the omega nomenclature* now is used widely even among the general public since the ingestion of fish oil now is fashionable. By comparison, the IUPAC (21) use of n-6, n-3 almost has fallen out of use! For a second example, since the present IUPAC system already has defined eicosanoids as icosanoids (22), any logical extension of this to a committee decision to use the term "HITEs" to define hydroxyeicosapolyenoic acids then would have to overturn 15 years of usage of HETEs!

It is hoped that this system of nomenclature will be as useful to others as it is to us and that it may serve as a basis for future developments.

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*This system designates chain length, number of double bonds and position of the double bond nearest to the terminal (omega; ω) methyl group (at opposite end from the α-carbon, which is linked to the terminal carboxyl group). It assumes skipped unsaturation and *cis*-geometry of double bonds. Thus, arachidonic acid is called 20:4ω6; eicosapentaenoic acid is 20:5ω3; docosahexaenoic acid is 22:6ω3.

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Editor's Note: Lipids is pleased to contribute to the growing dialogue on eicosanoid nomenclature and invites additional thoughts and comments.

Hydroxylation of Fatty Acids and Alcohols by Hepatic Microsomal Cytochrome P-450 System from the Mongolian Gerbil[1]

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The liver microsomes of the Mongolian gerbil *Meriones unguiculatus* catalyzed the hydroxylation of various saturated fatty acids (C_8-c_{18}), alcohols (C_{12} and C_{16}) and hydrocarbon (C_{12}) to the corresponding ω- and (ω-1)-hydroxy derivatives. Lauric acid was hydroxylated most effectively among saturated fatty acids and the order of activity as hydroxylation substrates was $C_{12} > C_{14} > C_{13} > C_{16} > C_{10} > C_{18} > C_8$. The specific activity of laurate hydroxylation (5.99 nmol/mg microsomal protein/min) in gerbil liver microsomes was higher than that observed in other species. 1-Dodecanol was also hydroxylated very effectively (4.58 nmol/mg microsomal protein/min) by gerbil liver microsomes, but in general the hydroxylation rates for fatty alcohols were much lower than those for the corresponding acids. It was found from both inhibitor and cofactor studies that the enzyme catalyzing the hydroxylation of fatty acids and alcohols in the liver microsomes of the Mongolian gerbil was a typical cytochrome P-450-linked monooxygenase, and at least two different cytochrome P-450 species were involved in the hydroxylation.

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Although mammals such as rat, mouse, guinea pig or rabbit are generally used in both basic and clinical research, some mammals other than these have been successfully developed as novel experimental animals (1—4) and used for a variety of specific research (5–7). In a previous paper (8), we described the substrate specificity and other properties of a fatty acid hydroxylase system in the liver microsomes of the house musk shrew (*Suncus murinus*), which has received attention as a possible laboratory animal because of such desirable traits as small size, rapid breeding and environmental flexibility. The cytochrome P-450 monooxygenase system in the shrew liver microsomes catalyzed specifically ω- and (ω-1)-hydroxylation of fatty acids to a high extent, and the correlation between the high rate of ω- (ω-)-hydroxylation of fatty acids and the energy metabolism in the shrew was discussed. The Mongolian gerbil (*Meriones unguiculatus*), which has also received attention as a possible laboratory animal for the same reasons, has been used for several studies on lipid biochemistry in recent years (7,9–12). However, no information is available concerning the fatty acid hydroxylating system in the gerbil liver. In this paper, the substrate specificity and kinetic properties of a cytochrome P-450-dependent monooxygenase system in the gerbil are described.

MATERIALS AND METHODS

Chemicals. ^{14}C-Labeled fatty acids, 1-dodecanol and hexadecane (sp acti 19–58 mCi/mmol) were purchased from Amersham International (Amersham, U.K.). Hexadecanol, labeled in the 1-position with ^{14}C, was prepared from [1-^{14}C]palmitic acid by $LiAlH_4$ reduction in dry ether at room temperature. Dodecane, labeled in the 1-position with ^{14}C, was chemically synthesized from [1-^{14}C]dodecanol. The alcohol was converted to [1-^{14}]dodecyl bromide (13), which in turn was refluxed with $LiAlH_4$ in dry tetrahydrofuran. Labeled dodecane was purified by silica gel chromatography and had a radiopurity of 99% by radio-gas chromatography. The ω-hydroxy derivatives of C_{10}, C_{12}, C_{14} and C_{18} fatty acids and (ω-1)-hydroxy derivatives of C_{12} and C_{14} fatty acids were synthesized chemically (14). ρ-Chloromercuribenzoate (PCMB), iodoacetic acid, KCN, NaN_3, menadione and 7,8-benzoflavone were purchased from Tokyo Kasei Chemical Co. (Tokyo). NADPH and NADH were from Oriental Yeast Co. (Tokyo). Cytochrome *c* (horse heart) was from Sigma Chemical Co. (St. Louis, MO). SKF 525A was a gift from Prof. M. Kusunose, Toneyama Institute for Tuberculosis Research, Osaka City University Medical School. Metyrapone was from Aldrich Chemical Co. (Milwaukee, WI). Sodium dodecylsulfate, sodium cholate, sodium deoxycholate, Triton X-100 and Tween 20 were from Tokyo Kasei. Emulgen 913 was supplied by Kao-Atlas Co. (Tokyo). The organic solvents were reagent grade and freshly distilled before use.

Preparation of gerbil liver microsomes. The Mongolian gerbils used in this study were obtained from our breeding colonies. Eight to 10 male gerbils (5 mo) weighing 51–66 g were used. The preparation of gerbil liver microsomes was caried out in the same manner as described for the preparation of frog liver microsomes (15). The microsomes were washed with 0.25 M sucrose and resuspended in the same medium. The protein concentration of the microsomal suspension was determined by the method of Lowry et al. (16). The cytochrome P-450 and cytochrome b_5 contents of the microsomes were determined by the methods of Omura and Sato (17) and Omura and Takesue (18), respectively. NADPH-cytochrome *c* reductase and HADH-cytochrome b_5 reductase activities were determined as described by Omura and Takesue (18,19).

Assays for hydroxylation of fatty acids, fatty alcohols and hydrocarbons. The standard incubations for the hydroxylation assay of fatty acids and 1-dodecanol contained microsomes (80–100 μg protein), 100 μmol potassium phosphate (buffer A, pH 7.5), 0.25 μmol NADPH and 30 nmol (4.7–5.3 × 10^4 cpm) of the appropriate ^{14}C-labeled fatty acids (added as the potassium salt) or 1-

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Abbreviations: PCMB, *p*-chloromercuribenzoate; SDS, sodium dodecylsulfate.

dodecanol. The final volume was 0.5 ml, and the incubation was 10 min at 37 C. Since the hydroxylase activity of 1-hexadecanol and hydrocarbons was low, the incubation mixture contained a greater amount (0.6–1.0 mg) of microsomes, 200 μmol buffer A, 0.5 μmol NADPH and 100–200 nmol of [1-^{14}C]hexadecanol or hydrocarbons (final volume 1.0 ml). The hydroxylase activities of the fatty acids and alcohols were assayed by the same method as described previously (20,21). ^{14}C-Labeled products (1- and 2-dodecanols) formed by the incubation of the microsomes with [1-^{14}C]dodecane were removed from silica gel with 7% ether in hexane while the substrate was eluted with 3% ether in hexane. The radioactivity in each fraction was determined by scintillation counting to determine the percentage of hydroxylation. All experiments were done in duplicate.

Isolation and identification of ω- amd (ω-1)-hydroxy fatty acids and alcohols. The several procedures used for isolation and identification of reaction products [ω- and (ω-1)-hydroxy fatty acids and alcohols] have been described in detail previously (20,21). The procedures used for radio-gas chromatographic analyses of the O-acetyl derivatives of hydroxy fatty acids and alcohols have already been described (8,20,21). ^{14}C-Labeled 1- and 2-dodecanols formed by the incubation of the gerbil liver microsomes with [1-^{14}C]dodecane were also analyzed by radio-gas chromatography.

RESULTS

Hepatic microsomal electron transport components and hydroxylase activity of Mongolian gerbil. Since no information is available on hepatic microsomal enzyme activities in the Mongolian gerbil, the cytochrome P-450 and cytochrome b_5 contents, NADPH-cytochrome *c* reductase and NADH-cytochrome b_5 reductase activities and laurate hydroxylase activity were measured (Table 1). Body weight, liver weight and microsomal protein of the Mongolian gerbils are also shown in Table 1.

TABLE 1

Hepatic Microsomal Electron Transport Components and Fatty Acid Hydroxylation in The Mongolian Gerbil, *Meriones uniguiculatus*[a]

Body weight (g)	59 ± 5
Liver weight (g/100 g body weight)	4.57 ± 0.53
Microsomal protein (mg/g liver)	2.41 ± 0.51
Cytochrome P-450	
nmol/mg microsomal protein	1.60 ± 0.16
nmol/g liver	3.92 ± 0.62
Cytochome b_5 (nmol/mg microsomal protein)	0.79 ± 0.01
NADH-cytochrome *c* reduction (μmol/mg microsomal protein/min)	0.18 ± 0.02
NADPH-cytochrome b_5 reduction (μmol/mg microsomal protein/min)	5.38 ± 0.29
Laurate hydroxylation[b]	
nmol/mg microsomal protein/min	5.99 ± 0.66
nmol/nmol P-450/min	4.03 ± 0.55

[a]Each value is the mean ± S.D. for 8–10 male Mongolian gerbils.
[b]The sum of ω- and (ω-1)-hydroxylaurate formed.

Substrate specificity for hydroxylation. After it was found that [1-^{14}C]laurate could be hydroxylated by gerbil liver microsomes, other saturated fatty acids and an unsaturated fatty acid (oleic acid) were tested as substrates, and the radioactive polar products formed enzymatically were analyzed by thin-layer chromatography and radio-gas chromatography. Table 2 shows the effect of substrate chain length on hydroxylation and distribution of ω- and (ω-1)-hydroxy products of fatty acids. Several points should be noted: laurate was the most active substrate. The ω/ω-1-hydroxylation ratio increased with increasing carbon chain length of fatty acids from 8 to 12. However, the ratio is constant (0.69–0.75) for the fatty acids rang-

TABLE 2

Substrate Specificity of Fatty Acid Hydroxylation by Gerbil Liver Microsomes

Saturated fatty acids (chain length)	Hydroxylation activity (nmol/mg microsomal protein/min)	Distribution of hydroxy isomers (%)		ω/ω-1-Hydroxylation
		ω	ω-1	
8	1.02 (17)[a]	7	93	0.075
10	2.70 (45)	16	84	0.19
12	5.99 (100)	51	49	1.04
13	4.79 (80)	43	57	0.75
14	4.91 (82)	43	57	0.75
16	3.17 (53)	41	59	0.69
18	1.20 (20)	43	57	0.75
18:1[b] (oleic acid)	3.83 (64)	43	57	0.75

[a]Values in parentheses are percentages of the specific activity of laurate hydroxylation.
[b]18:1 gave unknown products (0.92 nmol/mg microsomal protein/min) besides ω- and (ω-1)-hydroxy isomers. The products were not counted in the hydroxylation activity of 18:1.

TABLE 3

Hydroxylation Activity with Various Substrates in Gerbil Liver Microsomes

Substrate	Relative hydroxylation activity	Distribution of hydroxy isomers (%)	
		ω	ω-1
1-Dodecanol	78	56	44
Dodecane	6	89	11
Lauric acid	100[a]	52	48
1-Hexadecanol	6	53	47
Hexadecane	0	—	—
Palmitic acid	53	41	59

[a]Actual hydroxylation activity: 5.42 nmol/mg microsomal protein/min.

ing in chain length from 13 to 18 carbons. The hydroxylation activity of oleic acid was higher than that of its saturated analog (stearic acid), but the ω/ω-1-hydroxylation ratio for oleate was the same as that for stearate. Since fatty acids were effectively hydroxylated by gerbil liver microsomes, analogs such as fatty alcohols and hydrocarbons were also tested (Table 3). The hydroxylation activity of 1-dodecanol (lauryl alcohol) was high, but the activity of dodecane and 1-hexadecanol was much lower than that of 1-dodecanol or lauric acid. Hexadecane was not hydroxylated by gerbil liver microsomes.

Properties of the microsomal laurate hydroxylation. Since it was found that laurate was the most favored substrate among several fatty acids and alcohols, various properties of laurate hydroxylase of gerbil liver microsomes were investigated. The rate of ω- and (ω-1)-hydroxylation of laurate was linear with microsomal protein concentrations up to 180 μg for ω-hydroxylation and 200 μg for (ω-1)-hydroxylation (Fig. 1) and with time for about 15 min for ω-hydroxylation and 25 min for (ω-1)-hydroxylation (Fig. 2). The variation of hydroxylation activity with pH was determined (Fig. 3), and pH optimum was

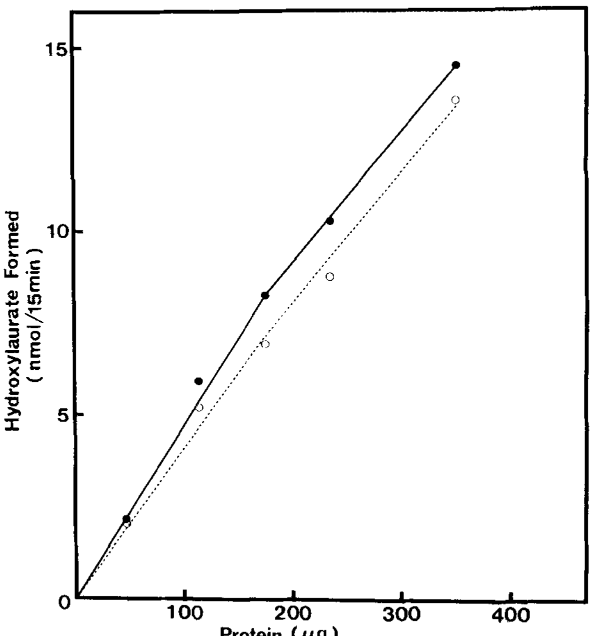

FIG. 1. Effect of protein concentration on rate of laurate hydroxylation by gerbil liver microsomes. ●, ω-Hydroxylation; ○, (ω-1)-hydroxylation.

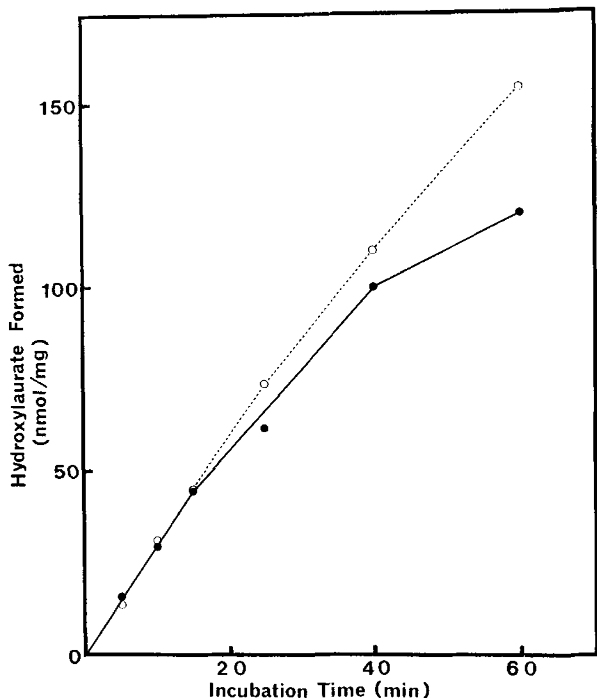

FIG. 2. Effect of time on rate of laurate hydroxylation by gerbil liver microsomes. The amount of microsomal protein was 98 μg. ●, ω-Hydroxylation; ○, (ω-1)-hydroxylation.

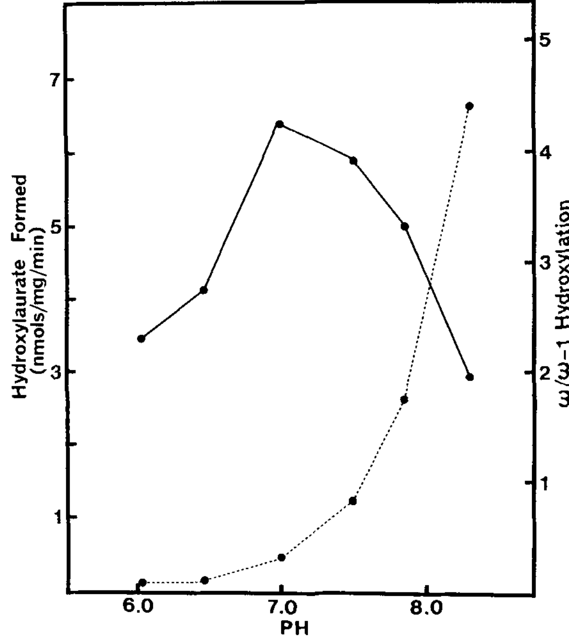

FIG. 3. Effect of pH on laurate hydroxylation by gerbil liver microsomes. The amount of microsomal protein was 114 μg. Solid line, total hydroxylation (the sum of ω- and [ω-1]-hydroxylation); dashed line, ω/ω-1-hydroxylation ratio.

TABLE 4

Effect of Reduced Pyridine Nucleotides on Laurate Hydroxylation by Gerbil Liver Microsomes

Reduced pyridine nucleotide	Relative hydroxylation activity	Distribution of hydroxy isomers (%)	
		ω	ω-1
NADH (50 μM)	8	8	92
NADH (100 μM)	16	8	92
NADH (500 μM)	25	26	74
NADPH (50 μM)	42	49	51
NADPH (500 μM)	100[a]	52	48
NADH (500 μM)			
+ NADPH (50 μM)	44	50	50
+ NADPH (500 μM)	102	54	46
None	4	—	—
NADPH (500 μM), anaerobic (helium)	9	37	63

[a]Actual hydroxylation activity: 6.70 nmol/mg microsomal protein/min.

observed at pH 7.0, but there was no significant difference in activity at pH 7.5. The ω/ω-1-hydroxylation ration increased with increasing pH.

The requirements of reduced pyridine nucleotides for laurate hydroxylation are shown in Table 4. NADPH was the preferred electron donor. NADH had little effect at 50 μM, but it showed 25% of the activity of NADPH (500 μM) at 500μM. When NADH was used for an electron donor, the percentage of the (ω-1)-hydroxy isomer was much greater than that of ω-hydroxy isomer. However, the percentage of the ω- and (ω-1)-hydroxy isomers was almost equal when NADPH was used as an electron donor. Although NADH was added together with NADPH, a synergistic effect of NADH was not observed. Molecular oxygen was also required for the hydroxylation of laurate.

Since laurate and 1-dodecanol has a similar hydroxylase activity, the data were plotted by the method of Lineweaver-Burk, and kinetic parameters for ω- and (ω-1)-hydroxy derivatives of lauric acid and 1-dodecanol were obtained. The apparent Km values for ω- and (ω-1)-hydroxylauric acid were 1.3×10^{-5} M and 3.5×10^{-5} M, respectively, and for ω- and (ω-1)-hydroxydodecanol were 9.1×10^{-5} M and 8.7×10^{-5} M, respectively. The Vmax for ω- and (ω-1)-hydroxylation of lauric acid were 2.84 and 2.67 nmol/mg microsomal protein/min, respectively, and for ω- for (ω-1)-hydroxylation of 1-dodecanol were 2.56 and 2.02 nmol/mg microsomal protein/min, respectively. Table 5 shows the effect of inhibitors on laurate hydroxylation activity. Carbon monoxide and PCMB strongly inhibited the total hydroxylation (the sum of ω- and [ω-1]-hydroxylation). Iodoacetate (0.1 mM), SKF 525A (1 mM), KCN (2 mM), menadione (0.01 mM) and NaN_3 (1 mM) inhibited total hydroxylation by 23%, 43%, 34%, 69% and 42%, respectively. Metyrapone did not inhibit the hydroxylation. Although iodoacetate, SKF 525A, KCN and menadione inhibited the total hydroxylation, they did not change the ω/ω-1-hydroxylation ratio (Table 5). On the other hand, PCMB and carbon monoxide were more inhibitory to the ω-hydroxylation of laurate than the (ω-)-hydroxylation, while NaN_3 was more inhibitory to the (ω-1)-hydroxylation than to the ω-hydroxylation. Although, 7,8-benzoflavone had almost no effect on the total hydroxylation, it showed a somewhat stimulatory effect on the ω-hydroxylation but an inhibitory effect on the (ω-1)-hydroxylation.

As detergents are often used for stimulation requirement of hydroxylation reaction by liver microsomes of various species, the effect of detergents on the laurate

TABLE 5

Effect of Inhibitors on Laurate Hydroxylation by Gerbil Liver Microsomes

Inhibitor	Laurate hydroxylation activity (nmol/mg microsomal protein/min)			ω/ω-1-Hydroxylation
	ω	ω-1	ω and ω-1	
None (control)	2.98 (100)[a]	2.44 (100)	5.42 (100)	1.22
Iodoacetate (0.1 mM)	2.14 (72)	2.06 (84)	4.20 (77)	1.04
SKF 525A (1 mM)	1.74 (58)	1.37 (56)	3.11 (57)	1.27
KCN (2 mM)	2.03 (68)	1.53 (63)	3.56 (66)	1.33
Metyrapone (0.1 mM)	2.99 (100)	2.55 (105)	5.54 (102)	1.17
Menadione (0.01 mM)	0.96 (32)	0.73 (30)	1.69 (31)	1.32
PCMB[b] (0.1 mM)	0.00 (0)	0.54 (22)	0.54 (10)	0.00
CO-O_2 (1:1, v/v)	0.26 (9)	0.63 (26)	0.89 (16)	0.41
CO-O_2 (9:1, v/v)	0.015 (0.5)	0.155 (6)	0.17 (3)	0.10
NaN_3 (1 mM)	2.22 (74)	0.90 (37)	3.12 (58)	2.47
7,8-BF[c] (0.1 mM)	3.50 (117)	1.73 (71)	5.23 (96)	2.02

[a]Values in parentheses are percentages of the specific activity to control.

[b]*p-Chloromercuribenzoate.*

[c]7,8-Benzoflavone.

TABLE 6

Effect of Detergents on Laurate Hydroxylation by Gerbil Liver Microsomes[a]

Detergent	Laurate hydroxylation activity (nmol/mg microsomal protein/min)		
	ω	ω-1	ω and ω-1
None (control)	2.98 (100)[b]	2.44 (100)	5.42 (100)
SDS[c]	0.33 (11)	1.84 (75)	2.17 (40)
Na Cholate	2.34 (79)	2.16 (89)	4.50 (83)
Na DOC[d]	1.87 (63)	2.47 (101)	4.34 (80)
Emulgen 913	1.98 (66)	2.74 (112)	4.72 (87)
Triton X-100	2.45 (82)	2.55 (105)	4.99 (92)
Tween 20	1.91 (64)	2.43 (100)	4.34 (80)

[a]Detergent concentration 0.005%.
[b]Values in parentheses are percentages of the specific activity to control.
[c]Sodium dodecylsulfate.
[d]Deoxycholate.

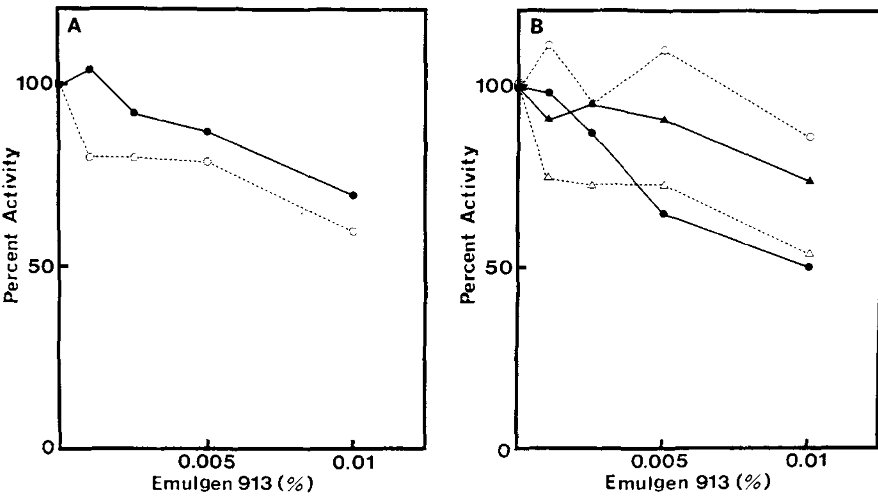

FIG. 4. Effect of Emulgen 913 on laurate hydroxylation activity of gerbil and rat liver microsomes. Experiment A: ●, gerbil; ○, rat. Experiment B: ●, ω-hydroxylation and ○, (ω-1)-hydroxylation by gerbil liver microsomes. ▲, ω-hydroxylation and △, (ω-1)-hydroxylation by rat liver microsomes. The amount of microsomal protein was 175 μg for both microsomes.

hydroxylation by gerbil liver microsomes was examined (Table 6). All the detergents were inhibitory to the total hydroxylation rather than stimulative to that. Sodium dodecylsulfate (SDS) was the most potent inhibitor. SDS and sodium cholate were inhibitory to the ω-hydroxylation of laurate than to the (ω-1)-hydroxylation. However, sodium deoxycholate, Emulgen 913, Triton X-100 and Tween 20 were inhibitory only to the ω-hydroxylation.

Since Emulgen 913 has been used for several studies on cytochrome P-450 (15,22,23), its effect on the laurate hydroxylase system in gerbil and rat liver microsomes was compared (Figs. 4A and 4B). Emulgen 913 was more inhibitory to the total activity of laurate hydroxylation by rat liver microsomes than to that by gerbil liver microsomes. However, Emulgen 913 had almost no inhibitory effect on the (ω-1)-hydroxylation by gerbil liver microsomes and the ω-hydroxylation by rat liver microsomes, but it was inhibitory to the ω-hydroxylation by gerbil liver microsomes and the (ω-1)-hydroxylation by rat liver microsomes.

DISCUSSION

Although several studies on lipid biochemistry of the Mongolian gebils have been reported in recent years (cf. the introduction), no information is available concerning their hepatic microsomal electron transport components or fatty acid–hydroxylating system. In this study, we in-

TABLE 7

Hydroxylation Activity of Fatty Acids by Various Microsomes (MS)

Enzyme source	Hydroxylation activity (nmol/nmol cytochrome P-450/min)				Reference
	C_{10}	C_{12}	C_{16}	C_{18}	
Gerbil liver MS	1.62 (100)[a]	4.03 (100)	1.89 (100)	0.71 (100)	—
Rat liver MS, (Wistar Imamichi)	—	2.80 (69)	—	—	(8)
Rabbit intestine MS	0.85 (52)	1.83 (45)	0.78 (41)	—	(26)
Shrew liver MS (*S. murinus*)	8.01 (494)	8.92 (221)	2.09 (111)	1.01 (142)	(8)
Frog liver MS	0.66 (41)	1.04 (26)	0.37 (20)	0.31 (44)	(20)

[a]Values in parentheses are percentages of the specific activity to gerbil liver microsomes.

vestigated various properties of the fatty acid– and alcohol-hydroxylating systems from the liver microsomes of the animals. The content of cytochrome P-450 (1.60α0.16 nmol/mg microsomal protein) in liver microsomes from the Mongolian gerbils is somewhat higher than that (0.87–1.22 nmol/mg microsomal protein) in male rat liver microsomes (24,25), but NADPH-cytochrome *c* reductase activity in the Mongolian gerbils is lower than that in male rat (24,25). The content of cytochrome b_5 in the gerbil liver microsomes is somewhat higher than that in male rat liver microsomes (24). Our present work demonstrated that gerbil liver microsomes, like the liver microsomes of other species, catalyzed the hydroxylation of fatty acids only at the ω- and (ω-1)-positions. Hydroxy isomers such as α-, β, (ω-2)- or (ω-3)-hydroxylaurates were not formed by gerbil liver microsomes. It was also demonstrated that the fatty acid hydroxylase in gerbil liver microsomes is a typical cytochrome P-450-dependent monooxygenase, because carbon monoxide, PCMB and SKF 525A are potent inhibitors of laurate hydroxylation, and molecular oxygen and NADPH were required for the hydroxylation. However, several characteristics of data in this study should be noted. First, the specific activity of fatty acid hydroxylation in gerbil liver microsomes is higher than that in other species except the shrew (*S. murinus*) (Table 7). We have reported already that hydroxylation activity of fatty acids in the liver microsomes of the shrew is much higher than that in other species (8). Thus, our previous and present results demonstrated that a cytochrome P-450 monooxygenase system in the liver microsomes of laboratory animals such as the shrew or the Mongolian gerbil catalyzed specifically the hydroxylation of fatty acids to a high extent. Secondly, the hydroxylation activity of 1-dodecanol (lauryl alcohol) is similar to that of lauric acid. Although the hydroxylation activity for fatty alcohols was much lower than that for the corresponding acids (21,27), it was found in this study that a cytochrome P-450 monooxygenase system in gerbil liver microsomes catalyzed specifically the hydroxylation of 1-dodecanol to a higher extent. Further experimental work is needed to definitively answer the question of why the hydroxylation activity of 1-dodecanol is markedly high in gerbil liver microsomes.

Since alcohol and aldehyde dehydrogenases are involved in the liver microsomes (21,28–30), a significant amount of fatty alcohols was comverted to fatty acids during the incubation of fatty alcohols with liver microsomes from various species (21,29). When 1-dodecanol or hexadecanol was incubated with gerbil liver microsomes, the conversion rate of 1-dodecanol to lauric acid was lower than that for liver microsomes of other species (data not shown), and 1-hexadecanol was not converted to palmitic acid. This suggests that dehydrogenase in gerbil liver microsomes is less active towards fatty alcohols.

Finally, the fact that the ω/ω-1-hydroxylation ration increased for the fatty acids ranging in chain length from 8 to 12 and is constant for fatty acids for which chain lengths were from 13 to 18 carbon atoms suggests that more than two cytochrome P-450 species in gerbil liver microsomes are involved in hydroxylation of fatty acids at ω- and (ω-1)-positions. The inhibitory study using PCMB, CO, NaN_3 and 7,8-benzoflavone suggests also the involvement of different cytochrome P-450 species in the ω- and (ω-1)-hydroxylation of lauric acid in gerbil liver microsomes because the ω/ω-1-hydroxylation ratios were changed. A similar result was reported for the laurate hydroxylation by frog liver microsomes (31). The fact that the detergents showed different effects on the ω- and (ω-1)-hydroxylation activity of laurate suggests also that gerbil liver microsomes contain different cytochrome P-450 species catalyzing the ω- and (ω-1)-hydroxylation of laurate. However, further experimental work is needed to elucidate the involvement of different cytochrome P-450 species in fatty acid hydroxylation. It has been reported previously that hepatic microsomal cytochrome P-450-dependent monooxygenases from different sources (15) or other enzyme systems (32) had different responses to detergents. A similar result was observed in our study; the hydroxylase system in gerbil liver microsomes is less susceptible to inhibition by Emulgen 913 in the assay of laurate hydroxylation than the hydroxylase system in rat liver microsomes. Thus,it was found in this study that the microsomal hydroxylase system in gerbil liver has several unique features.

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Intake of Different Eicosapentaenoic Acid–Containing Lipids and Fatty Acid Pattern of Plasma Lipids in the Rats

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The ethyl ester of eicosapentaenoic acid (EPA) is the only pure EPA-containing lipid available in bulk for oral administration. However, there is doubt as to whether EPA ethyl ester can efficiently increase the plasma levels of EPA in comparison with the ability of other kinds of EPA-containing lipids to do so. Therefore, two other kinds of EPA-containing lipids were prepared to study the efficiency of oral administration of those lipids for increasing the EPA content in plasma phospholipids and cholesteryl esters. EPA-containing lipids which were investigated were [A] 1,2,3-trieicosapentaenoyl-glycerol, [B] 2-eicosapentaenoyl-phosphatidylcholine and [C] ethyl ester of EPA. An adjusted amount of lipids [A], [B] and [C] was administered to rats through a gastric tube for 4 days (the first experiment) or for 10 days (the second experiment), and the fatty acid composition of plasma phospholipids and cholesteryl esters was determined. In the first experiment, there were no significant differences in the efficiency for increasing EPA levels in either phospholipids or cholesteryl esters among the lipids. In the second experiment, the EPA levels of both plasma phospholipids and cholesteryl esters of rats administered ethyl ester of EPA were significantly higher than those of rats administered 2-eicosapentaenoyl-phosphatidylcholine. The EPA levels of the rats administered 1,2,3-trieicosapentaenoyl-glycerol were between the levels of the two groups mentioned above, but the differences in the EPA levels were not significant. Although an ethyl ester–type molecule is not a naturally occurring lipid, ethyl ester of EPA is equal to 1,2,3-trieicosapentaenoyl-glycerol and appears to be superior to 2-eicosapentaenoyl-phosphatidylcholine as to the efficiency for increasing EPA levels in total plasma phospholipids and plasma cholesteryl esters.

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Beginning with epidemiological studies by Bang and Dyerberg in Greenland (1,2), many papers have indicated that fish oil has antiatherosclerotic features (3–10). The active components in fish oil are said to be ω3 fatty acids, among which eicosapentaenoic acid (EPA) has been most investigated. Some clinical trials with ethyl ester (EE) of EPA have been performed (11,12), and others are underway to develop EPA as a drug. At present, EE type is the only pure (~ 90%) EPA-containing molecule available in bulk for oral administration experiments on humans or other animals.

Because EE was not a natural product, we thought that it might not be absorbed very well and that some other natural types of lipid molecule containing EPA might be better in terms of absorption and utilization efficiency. The purpose of this study was to compare the efficiency for increasing EPA levels in plasma phospholipids (PL) and cholesteryl esters (CE) of different EPA-containing lipid molecules.

MATERIALS AND METHODS

Materials. Feeding experiments were performed using the following lipids: [A] 1,2,3-trieicosapentaenoyl-glycerol (EPA-TG); [B] 2-eicosapentaenoyl-phosphatidylcholine (EPA-PC); and [C] ethyl ester of EPA (EPA-EE). EPA-EE was obtained as described previously (13) with slight modifications. EPA-TG was made by chemical condensation of glycerol and 90% pure free EPA, which was obtained by hydrolysis of EPA-EE. EPA-PC was also made by chemical condensation of free EPA and enzymatically prepared lysophosphatidylcholine from soybean PC. Incomplete reaction products of these materials were separated by silica gel columns. Fatty acid analysis of the lipids mentioned above is shown in Table 1. Lipids [A] to [C] contained α-tocopherol (0.2%, w/w) as an antioxidant.

Feeding experiments 1 and 2. In Experiment 1, 18 male Wistar rats weighing about 500 g, which had been fed on the lipid-free powder diet (Funabashi Farm, Chiba) for 20 days, were divided into four groups (A, B, C and D). All rats were given free access to the same diet throughout the experiment. Rats of groups A, B and C were administered EPA (400 mg/kg/day) in the form of a lipid suspension (~ 5 ml) through a gastric tube for four days. The suspensions of groups A, B and C were suspensions of EPA-TG, EPA-PC and EPA-EE, respectively. Rats of group D were administered water (~ 5 ml) containing no lipids. Blood samples were obtained from the abdominal aorta under ether anesthesia one day after the last administration of the lipid suspension or water. Experiment 2 was performed in the same way as experiment 1, except that rats were administered EPA or water for 10 days.

Platelet aggregation and fatty acid analysis. A platelet aggregation study was performed with a PAT-4M aggregometer (Niko Bioscience, Tokyo) on platelet-rich plasma obtained by centrifugation of citrated blood with

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Abbreviations: AA, arachidonic acid; CE, cholesteryl esters; EE, ethyl ester; EPA, eicosapentaenoic acid; PC, phosphatidylcholine; PL, phospholipids; TG, triglycerides.

TABLE 1

Fatty Acid Composition of Lipids Used in the Experiments (mol%)

Lipids	Fatty acids								
	16:0	18:0	18:1	18:2ω6	18:3ω3	18:4ω3	20:4ω6	20:4ω3	20:5ω3 (EPA)
[A] EPA-TG						2	5	2	89
[B] EPA-PC									
Position 1	26	7	20	46	2				
Position 2						2	5	2	89
[C] EPA-EE						2	5	2	89

EPA-TG, 1,2,3-trieicosapentaenoyl-glycerol; EPA-PC, 2-eicosapentaenoyl-phosphatidylcholine; EPA-EE, ethyl ester of eicosapentaenoicd acid.

TABLE 2

Percentage Composition of Major Fatty Acids in Plasma Total Phospholipids after Forced Administration of EPA-Containing Lipids or Water for 4 Days (Experiment 1)[a]

Groups	n	Fatty acids (mol % ± SD)						
		16:0	18:0	18:1	18:2ω6	20:4ω6	20:5ω3	22:6ω3
[A] EPA-TG	4	32.0	23.2[b]	12.7	7.6	11.1	1.0[c]	6.8
		(3.2)	(1.4)	(1.3)	(2.1)	(2.3)	(0.4)	(0.5)
[B] EPA-PC	4	32.2[b]	24.2	11.7	6.9	8.0	1.5[d]	7.0
		(2.4)	(2.9)	(3.3)	(2.4)	(0.9)	(0.3)	(1.0)
[C] EPA-EE	4	30.4	26.4	11.9	5.8	10.0	1.6[d']	6.0
		(3.1)	(1.7)	(0.5)	(2.3)	(1.3)	(0.3)	(0.8)
[D] Water	5	26.5[b]	28.8[b]	13.6	7.2	11.8	0.2[c,d,d']	5.0
		(1.8)	(2.2)	(1.7)	(1.3)	(3.1)	(0.1)	(1.8)

EPA-TG, 1,2,3-trieicosapentaenoyl-glycerol; EPA-PC, 2-eicosapentaenoyl-phosphatidylcholine; EPA-EE, ethyl ester of eicosapentaenoicd acid.

[a]Rats were administered adjusted amounts of a suspension containing one of the lipids shown above (400 mg of EPA/kg/day) or water through a gastric tube for four days, while they were fed on a synthetic lipid-free diet. SD is shown in the parentheses. Values with the same superscript in common are significantly different from each other.

[b]$p < 0.05$.

[c]$p < 0.01$.

[d(d')]$p < 0.001$.

1/10 volume of 3.8% citrate, using collagen (10 μg/ml) and ADP (5 μM) as aggregants. For fatty acid analysis, plasma was obtained from EDTA-anticoagulated blood. The fatty acid composition in plasma total PL and CE was measured according to the method of Lasserre (14), with slight modifications. Briefly, plasma lipids were extracted with chloroform/methanol (2:1, v/v). Lipids were then separated using a Bond Elut column (Analytichem International, Harbor City, CA) (15) to get the PL fraction. The CE fraction was further separated on a silica gel plate. Fatty acids in these fractions were transmethylated by BF_3. An Advance-DS capillary column, measuring 0.24 mm × 25 m (Shinwakagaku, Kyoto), was used to analyze fatty acid methyl ester. The column was attached to a GC7A gas choromatograph (Shimadzu, Kyoto). The injection was at 250 C; separation with N_2 gas as a carrier (40 ml/min) was isothermal at 200 C; detection was by flame ionization.

Total PL and CE were measured enzymatically with a Hitachi Autoanalyzer 716 (Tokyo). Analysis of variance and Scheffe's s-test were used to detect significant variations among the groups and to compare two dietary groups, respectively. The difference between experiments 1 and 2 within the same dietary group was analyzed by Student's t-test. Data were expressed as mean ± SD.

RESULTS

Feeding experiment 1. One of the group C rats died on the third day of the diet because of trauma in the esophagus during the intubation. Otherwise, there were no significant changes in body weight during the experiment. The percentage composition of major fatty acids in plasma total PL and CE is shown in Tables 2

TABLE 3

Percentage Composition of Major Fatty Acids in Plasma Cholesteryl Ester after Forced Administration of EPA-Containing Lipids or Water for 4 Days (Experiment 1)[a]

Groups	n	Fatty acids (mol % ± SD)						
		16:0	16:1	18:1	18:2ω6	20:4ω6	20:5ω3	22:6ω3
[A] EPA-TG	4	7.1[b]	7.2	6.9	7.8	58.4	9.4[c]	2.2
		(1.7)	(2.1)	(1.2)	(0.1)	(8.8)	(4.6)	(0.2)
[B] EPA-PC	4	6.8	10.2	8.5	8.4	46.2	15.2[d]	2.4
		(0.7)	(2.7)	(1.3)	(0.8)	(6.6)	(2.5)	(0.5)
[C] EPA-EE	4	5.9	9.4	6.9	7.9	50.8	14.8[d']	2.1
		(0.3)	(1.5)	(0.6)	(0.5)	(3.8)	(2.5)	(0.2)
[D] Water	5	5.0[b]	12.0	7.0	7.4	56.7	1.6[c,d,d']	1.9
		(0.6)	(2.9)	(2.3)	(1.3)	(7.7)	(0.4)	(0.3)

EPA-TG, 1,2,3-trieicosapentaenoyl-glycerol; EPA-PC, 2-eicosapentaenoyl-phosphatidylcholine; EPA-EE, ethyl ester of eicosapentaenoicd acid.

[a]Rats were administered adjusted amounts of a suspension containing one of the lipids shown above (400 mg of EPA/kg/day) or water through a gastric tube for four days, while they were fed on a synthetic lipid-free diet. SD is shown in the parentheses. Values with the same superscript in common are significantly different from each other.

[b]$p < 0.05$.

[c]$p < 0.01$.

[d(d')]$p < 0.001$.

TABLE 4

Percentage Composition of Major Fatty Acids in Plasma Total Phospholipids after Forced Administration of EPA-Containing Lipids or Water for 10 Days (Experiment 2)

Groups	n	Fatty acids (mol % ± SD)						
		16:0	18:0	18:1	18:2ω6	20:4ω6	20:5ω3	22:6ω3
[A] EPA-TG	4	32.2	24.6	12.0	8.3	9.0	1.7[c]	6.7
		(2.4)	(2.6)	(1.8)	(1.3)	(0.9)	(0.3)	(2.0)
[B] EPA-PC	4	30.4	25.1	11.0	↑10.9[a,b]	↑10.6	1.1[b,b']	6.8
		(3.3)	(1.1)	(1.1)	(0.7)	(1.5)	(0.5)	(1.1)
[C] EPA-EE	5	29.4	23.9	14.0	8.0[a]	8.7	2.1[b,c']	6.8
		(2.3)	(8.7)	(1.9)	(1.6)	(1.3)	(0.3)	(0.6)
[D] Water	5	28.5	25.7	11.6	7.1[b]	↑16.8*	0.03[b',c,c']	5.7
		(1.7)	(3.4)	(2.9)	(0.8)	(2.2)	(0.1)	(1.0)

SD is shown in parentheses. Values with the same superscript in common are significantly different from each other. Arrows denote significant increase compared to the counterpart in Table 2; ↑, $p < 0.05$. EPA-TG, 1,2,3-trieicosapentaenoyl-glycerol; EPA-PC, 2-eicosapentaenoyl-phosphatidylcholine; EPA-EE, ethyl ester of eicosapentaenoicd acid.

[a]$p < 0.05$

[b,(b')]$p < 0.01$.

[c,(c')]$p < 0.001$.

*, Different from groups A, B and C with $p < 0.001$.

and 3, respectively. There were no differences in plasma PL and CE levels among the four groups (data not shown). There were no significant differences in the major fatty acid composition among groups A, B and C either in plasma PL or CE. There were no significant differences in platelet aggregation induced either by collagen or by ADP among the groups (data not shown).

Feeding experiment 2. There was a slight increase in body weight (~ 10 g) in groups A, C and D, but no changes in group B. There were no significant differences in the changes of body weight among the four groups. The percentage composition of major fatty acids in plasma total PL and CE is shown in Tables 4 and 5, respectively. There were no differences in plasma PL and CE levels among the four groups (data not shown). In plasma total PL fraction for Groups A, B and C, 18:2 fatty acid was significantly higher in group B than in group C; EPA was significantly higher in group C than

TABLE 5

Percentage Composition of Major Fatty Acids in Plasma Cholesteryl Ester after Forced Administration of EPA-Containing Lipids or Water for 10 Days (Experiment 2)

		Fatty acids (mol % ± SD)						
Groups	n	16:0	16:1	18:1	18:2ω6	20:4ω6	20:5ω3	22:6ω3
[A] EPA-TG	4	7.2	7.4	7.8	7.5	49.5[c]	15.8	2.0
		(0.4)	(1.3)	(2.1)	(0.8)	(3.3)	(2.2)	(0.6)
[B] EPA-PC	4	7.1	↓ 6.2	7.5	9.8[a]	55.6[a,b]	10.7[a]	1.8
		(0.7)	(0.8)	(1.0)	(0.7)	(5.8)	(3.4)	(0.4)
[C] EPA-EE	5	6.9	9.2	↑↑8.9	7.6	45.0[a,c']	18.8[a]	2.1
		(1.2)	(3.1)	(0.8)	(1.7)	(5.4)	(2.8)	(0.3)
[D] Water	5	6.0	↓ 5.8	7.5	6.4[a]	↑ 70.2[b,c,c']	↓↓ 0.6*	↓1.5
		(0.8)	(2.5)	(1.0)	(0.6)	(4.7)	(0.4)	(0.1)

SD is shown in parentheses. Values with the same superscript in common are significantly different from each other. Arrows denote significant increase or decrease compared to the counterpart in Table 3; ↑ (↓), $p < 0.05$; ↑↑ (↓↓), $p < 0.01$. EPA-TG, 1,2,3-trieicosapentaenoyl-glycerol; EPA-PC, 2-eicosapentaenoyl-phosphatidylcholine; EPA-EE, ethyl ester of eicosapentaenoicd acid.
[a] $p < 0.05$
[b] $p < 0.01$.
[c,(c')] $p < 0.001$.
*, Different from groups A, B and C with $p < 0.001$.

TABLE 6

Summary of Short-term Feeding Experiments in Rats and Humans[a]

		EPA				
Ref	Source	Amount in diet	Daily dose/kg[b]	Feeding period[c]	Used Fraction for fatty acid analysis[d]	Changes in EPA (%)
Rat experiments						
16	Fish oil	1% fish oil	100 mg/kg	2 wk	Plasma PL	0 to 3.46
		5% fish oil	500 mg/kg	2 wk	Plasma PL	0 to 8.53
17	MaxEPA	10% fish oil	120 mg/kg	2 wk	Total plasma lipids	0 to 8.4
18	EPA-EE	3%EPA-EE	3000 mg/kg	10 days	Total serum lipids	0 to 11.5
Human trials						
19	MaxEPA	20 ml	~ 50 mg/kg	2 wk	Plasma PC	1.5 to 6.7
20	Mackerel	2 cans	~ 30 mg/kg (2.2 g EPA/day)	2 wk	Serum CE	1.5 to 10.9
	Herring	2 cans	~ 14 mg/kg (1.0 g EPA/day)	2 wk	Serum CE	1.9 to 8.2
21	Mackerel	750 g	140-210 mg/kg (10–15 g EPA/day)	3 days	Plasma PL	1.1 to 12.2
22	Cod liver oil	15 ml	~ 19 mg/kg	2 wk	Plasma PL	0.8 to 3.8
					Plasma CE	0.7 to 3.1
23	EPA-EE	6 g	~ 90 mg/kg	6 days	Plasma PL	1.4 to 7.6

[a]Feeding experiments, the periods of which are not more than 2 weeks, are listed.
[b]Daily dose/kg was estimated by assuming that rats ate about 1/10 as much diets as their own body weight or that the human body weight was 70 kg.
[c]Some experiments have a longer feeding period (not shown in this table) besides the listed period.
[d]Some fractions other than PL and CE are omitted.
EPA, eicosapentaenoic acid; PL, phospholipids; EE, ethyl ester; PC, phosphatidylcholine; CE, cholesteryl ester; PL phospholipids.

in group B. In the plasma CE fraction for groups A, B and C, arachidonic acid (AA) was significantly higher in group B than in group C; EPA was significantly higher in group C than in group B. There were no significant differences in platelet aggregation induced either by collagen (group A, 58.7 ± 10.7; B, 61.0 ± 6.2; C, 59.5 ±2.5; D, 61.0 ± 7.3, expressed as % maximum aggregation) or by ADP (A, 56.0 ± 4.6; B, 60.5 ± 5.9; C, 54.0 ± 6.9; D, 58.7 ± 4.0).

DISCUSSION

Details of some feeding experiments with fish oil or EPA are shown in Table 6. In all the experiments listed the blood EPA levels were determined within 2 wk of the start of EPA feeding. The increases in the EPA levels in these experiments with rats (16–18) were all bigger than that of the present study. This discrepancy may be partially explained by the difference in feeding periods and used fractions for fatty acid analysis. However, the major difference seems to consist in the materials used for feeding EPA. We administered pure EPA suspensions. In the experiments of references 16 and 17, fish oil was used, and it contained 25.6% and 9% of DHA respectively. Because DHA is known to increase the plasma PL levels of EPA, probably by the retroconversion of DHA (23), direct comparison of EPA contained in fish oil and pure EPA for ability to increase the blood levels of EPA is difficult. Although a pure EPA-EE was used in case of reference 18, the estimated daily dose of EPA was too high (~3000 mg/kg) to compare with ours (400 mg/kg). It is also possible that EPA mixed in a diet (16–18) may be absorbed and utilized better than that of water suspension, which is administered through a gastric tube like in the present study, although an accurate amount of EPA can be administered in this method.

In experiment 1, three EPA-containing lipids produced essentially the same effect on the composition of major fatty acids either in the fraction of plasma PL (Table 2) or in that of CE (Table 3). As a result of the extension of EPA administration to 10 days, the EPA levels of both plasma PL and CE of group C became significantly higher than those of group B (Tables 4 and 5). The EPA levels of group A were between the levels of the two groups mentioned above. Although an ethyl ester–type molecule is not a naturally occurring lipid, ethyl ester of EPA is equal to EPA-TG and appears to be superior to EPA-PC with regard to the efficiency for increasing EPA levels in plasma PL and CE in rats.

Among the EPA-containing lipids used in the present study, EPA-PC was the only lipid that contained 18:2 fatty acid, and the content of this fatty acid was about half as much as that of EPA in the lipid (Table 1). Therefore, the increment in 18:2 fatty acid (Table 4) and AA (Table 5), a converted fatty acid of 18:2 fatty acid, in group B probably caused a depression of the EPA levels compared to group C.

It is known that platelet aggregation of rats is seldom depressed by the administration of fish oil or EPA; only one out of five papers reported depressed platelet aggregation by EPA feeding (24). Therefore, it is not surprising that in the present two experiments we could not observe any significant depression of platelet aggregation by EPA-containing lipids.

As can be seen from Table 6, there is an important species difference between rats and humans. The EPA levels in humans can be increased with much smaller doses of EPA than in rats. Therefore, the efficiency of EPA-EE for increasing EPA levels in plasma in humans by comparison with other EPA-containing lipids remaining to be studied.

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Properties of Monoacylglycerol Lipase in Rabbit Aorta

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Monoacylglycerol lipase activity was characterized in a soluble preparation from rabbit aorta (intima-media) obtained by combining a 100,000 × g supernatant fraction with activity solubilized from the 100,000 × g precipitate fraction by treatment with Triton X-100. Rates of hydrolysis with 1-monoolein and 2-monoolein substrates were nearly identical. 1-Monoolein was a competitive inhibitor (Ki 65 μM) of 2-monoolein hydrolysis. 2-Monoolein and 2-monopalmitin were both hydrolyzed more rapidly than 2-monoarachidonin. Lipase activity measured with a 2-monoolein substrate was inhibited by the addition of oleate, NaF and $CaCl_2$ to the assay. Preincubation of the lipase preparation with p-bromophenacyl bromide resulted in a potent inhibition of lipase activity; this inhibition could be prevented by dithiothreitol.
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The receptor-mediated breakdown of phosphatidylinositol and the polyphosphoinositides plays an important role in signal transduction (1-3). The diacylglycerol (DG) generated by the phosphoinositide-specific phospholipase C reaction can activate protein kinase C and thus influence cell function (2,3). The metabolism of released DG will result in the termination of this signal. DG can be phosphorylated to form phosphatidic acid (kinase activity) or be hydrolyzed (lipase activity) to release the fatty acids from the *sn*-1 and *sn*-2 positions (2). Since arachidonic acid is the predominant fatty acid in the *sn*-2 position of the DG released from the phosphoinositides, the lipase pathway can provide the arachidonic acid precursor necessary for the synthesis of prostanoids, which are also capable of regulating cell function (1,2). Prostanoids such as PGI_2 are important products of arachidonic acid metabolism in blood vessels (4), where they contribute to the regulation of interactions between platelets and the vessel wall (5).

Recently, we have measured DG kinase and lipase activities in aorta (6) and in coronary and brain microvessels (6,7). The hydrolysis of DG by particulate and soluble subcellular fractions from rabbit aorta (intima-media) proceeded by an ordered two-step reaction sequence in which the fatty acid at the *sn*-1 position of the 1,2-DG substrate was released first, followed by the hydrolysis of the fatty acid from the 2-monoacylglycerol (2-MG) intermediate (6). Evidence for this reaction sequence has also been obtained with platelets (8,9) and decidua vera tissue (10). Since it is a 2-MG lipase activity that actually catalyzes the release of arachidonic acid in the phospholipase C-DG lipase pathway, the objective of the present investigation was to further characterize this enzyme, with respect to substrate specificity and regulation, in preparations from rabbit aorta.

MATERIALS AND METHODS

Materials. [^{3}H]Triolein (glycerol tri[9,10-^{3}H(N)]oleate), [^{14}C]tripalmitin (glycerol tri[1-^{14}C]palmitate) and 1-stearoyl-2[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine were purchased from Amersham Canada (Oakville, Ontario). 1-[1-^{14}C]Monoolein (1-[^{14}C]monooleoylglycerol) was obtained from RoseChem (Los Angeles, CA). 1-Palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol was synthesized as described by Hee-Cheong et al. (7). Unlabeled lipids (1-stearoyl-2-arachidonoyl-*sn*-glycerol, 1-palmitoyl-2-oleoyl-*sn*-glycerol, 1-monoolein, 2-monoolein, 2-monopalmitin, oleic acid and pig brain phosphatidylserine) were obtained from Serdary Research Laboratories (London, Ontario). Essentially fatty acid-free bovine albumin was purchased from Sigma Chemical Co. (St. Louis, MO).

Enzyme preparation. The intima-media fraction was dissected from rabbit aortas (Pel-Freez Biochemicals, Rogers, AR) and homogenized as detailed previously (6). Following centrifugation at 1,000 × g, the low-speed supernatant fraction was centrifuged at 100,000 × g to yield soluble and particulate fractions. The particulate fraction was resuspended in a solution consisting of 0.25 M sucrose, 1 mM EDTA, 1 mM DTT (dithiothreitol) and 10 mM HEPES (N-2-hydroxyethyl-piperazine-N′-2-ethane sulfonic acid), pH 7.5. Triton X-100 was added to a final concentration of 1 mM and after 5 min at 4 C, the sample was sonicated for 30 sec and centrifuged at 100,000 × g for 60 min. This supernatant fraction was added to the first soluble fraction, and the combined solution was concentrated by vacuum dialysis. The protein content of the concentrated fraction was usually 5-6 mg/ml (11).

Preparation of substrates. 2-[^{3}H]Monoolein (2-[^{3}H]monooleoylglycerol) was isolated following the incubation of [^{3}H]triolein with pancreatic lipase as described by Severson and Hee-Cheong (6). The radiolabeled 1-monoolein and 2-monoolein compounds were subjected to thin layer chromatography (TLC) on glass Silica Gel G plates impregnated with 10% (w/w) boric acid (12) to monitor acyl migration of the label. After development in a solvent system consisting of chloroform/methanol (98:2, v/v), the monoacylglycerols were clearly separated with Rf values of 0.43 and 0.66 for the 1- and 2-monooleins, respectively. The synthesized 2-[^{3}H]monoolein was quite stable; 82% of the radioactivity was present as 2-monoolein when monitored after three months of storage in hexane at −20 C. 2-[^{14}C]Monopalmitin (2-[^{14}C]monopalmitoylglycerol) was prepared from [^{14}C]tripalmitin following incubation with pancreatic lipase (6). The 2-monoolein and 2-monopalmitin substrates were adjusted to a specific activity of 1000 DPM/nmol with the addition of the appropriate unlabeled monoacylglycerol. 2-[^{14}C]Monoarachidonin (2-[^{14}C]monoarachidonoylglycerol) was synthesized in two steps. First, 1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycerol was prepared by the hydrolysis of 1-stearoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine by phos-

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Abbreviations: DG, diacylglycerol; 2-MG, 2-monoacylglycerol; DTT, dithiothreitol; TLC, thin layer chromatography; BPB, p-bromophenacyl bromide; PMSF, phenylmethylsulfonyl fluoride

pholipase C (7). The specific activity of the radiolabeled DG was adjusted to 1000 DPM/nmol with the addition of unlabeled 1-stearoyl-2-arachidonoyl-*sn*-glycerol, and 2-[^{14}C]monoarachidonin was then isolated following incubation with pancreatic lipase (6).

Enzyme assays. Emulsions of the monoacylglycerol substrates were prepared by sonication essentially as described by Severson and Hee-Cheong (6). Unless noted otherwise, the standard 2-MG lipase assay was performed as follows. An appropriate quantity of 2-[^{3}H]monoolein (4 nmol/μl of hexane) was dried under N_2 and then sonicated for 30 sec (75 W; BraunSonic 1510 sonicator) in 0.005% Triton X-100. A substrate mixture was prepared by diluting the emulsion with 0.25 M phosphate buffer (pH 7). Lipase assays (0.5 ml final volume) contained 80 μM 2-[^{3}H]monoolein, 50 mM sodium phosphate (pH 7), 0.001% Triton X-100 and appropriate quantities of the soluble lipase preparation. Incubations were terminated after 20 min at 37 C, and the quantity of released [^{3}H]oleate was measured by scintillation spectrometry following liquid-liquid partitioning (6). A unit of lipase activity was arbitrarily defined as the amount of enzyme that catalyzed the release of 1 nmol of fatty acid in 1 hr at 37 C. In experiments where inhibitors were added to the lipase assay, the specific activity of the 2-monoolein substrate was increased to 2500 DPM/nmol.

Monoacylglycerol kinase activity was measured by adding 5 mM ATP and 10 mM $MgCl_2$ to the lipase assay mixture described above. After an incubation of 20 min, the assay was terminated by the addition of butanol (13). Following centrifugation, an aliquot of the clear upper phase (0.75 ml) was dried under N_2, resuspended in chloroform and then applied to glass TLC plates (Sil G-25). Lysophosphatidic acid was isolated after development of the plates with a solvent system (13) consisting of chloroform/methanol/7M ammonia (60:35:5, v/v/v).

Diacylglycerol kinase activity in particulate fractions from aorta (6) was measured as described previously (7), with the exception that the 1-palmitoyl-2-[1-^{14}C]oleoyl-*sn*-glycerol substrate was prepared as a co-sonicate with phosphatidylserine (14); the final concentration of phosphatidylserine in the assay was 0.1 mM.

RESULTS

When the initial low-speed (1000 × g) supernatant fraction obtained from rabbit aorta (intima-media) homogenates was centrifuged at 100,000 × g, approximately half of the total recovered 2-MG lipase activity was particulate (6). Sonication of the resuspended particulate fraction in the presence of the nonionic detergent Triton X-100 resulted in the recovery of 65 ± 1% (mean ± SEM; n = 3) of the activity in the supernatant after centrifugation at 100,000 × g. The final specific activity for 2-MG lipase activity after combination of the first and second supernatant fractions and concentration was 325 Units/mg protein (mean of seven preparations), an increase of about 1.8-fold over the activity in the first high-speed supernatant fraction (6). Further attempts to purify the 2-MG lipase by a variety of chromatographic techniques (DEAE-Sephacel, CM-Sephadex, heparin-Sepharose, Ultrogel AcA-34) were unsuccessful in producing a significant increase in lipase specific activity. Therefore, characterization of the enzyme proceeded with this soluble fraction.

The positional specificity of aortic 2-MG lipase activity was examined by comparing rates of hydrolysis in assays with 1- and 2-monoolein substrate preparations (Fig. 1). The reaction rate for both substrates was reasonably linear with respect to time; the rate of hydrolysis of the 1-monoolein substrate was slightly greater than the hydrolysis of 2-monoolein. The presence of 1-monoolein resulted in a concentration-dependent inhibition of the hydrolysis of 2-monoolein (Fig. 2); a concentration of 200 μM 1-monoolein produced a 50% inhibition of aortic 2-MG lipase. The kinetic mechanism for the inhibition of 2-MG lipase activity by 1-monoolein was investigated (Fig. 3). The apparent Km for lipase activity measured with the 2-monoolein substrate was 53 μM. In the presence of 1-monoolein (200 μM or 500 μM), 2-MG lipase activity was inhibited in a competitive fashion (Fig. 3), with a calculated Ki of 65 μM. 1-Monoolein has been reported to inhibit DG kinase activity from pig brain (14). As shown in Figure 2, 1-monoolein also inhibited DG kinase activity in aortic microsomes; the inhibition of DG kinase activity was slightly greater than the inhibition of 2-MG lipase activity at all concentrations of 1-monoolein. No MG kinase activity could be detected in the soluble fraction used in this investigation when lipase assay incubations were modified by the addition of ATP and $MgCl_2$.

The substrate specificity of the lipase preparation for 2-monoacylglycerols was investigated next. As indicated in Figure 4, the relative rates of hydrolysis were 2-monopalmitin > 2-monoolein > 2-monoarachidonin. When a number of assay incubations where time or protein were varied were all considered (n = 14), lipase activity with 2-monoarachidonin as substrate was 71 ± 2% of the activity measured with 2-monoolein.

The sensitivity of aortic 2-MG lipase to product inhibition by oleate is shown in Figure 5. The addition of oleate directly to the assay produced a concentration-dependent inhibition of lipase activity; 50% inhibition occurred at

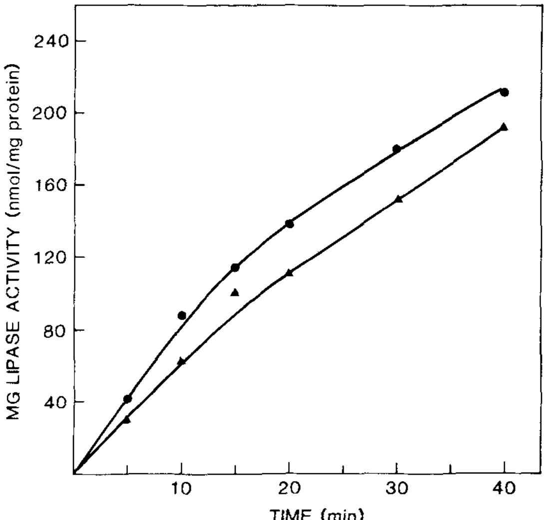

FIG. 1. Positional substrate specificity for aortic 2-monoacylglycerol (MG) lipase activity. The hydrolysis of 1-[^{14}C]monoolein (●) and 2-[^{3}H]monoolein (▲) substrates was determined at the indicated incubation times.

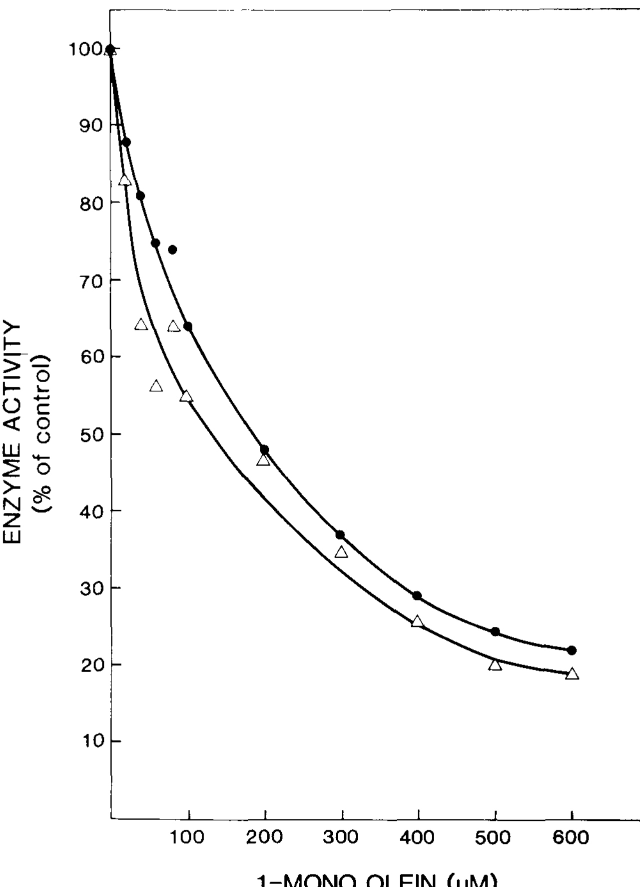

FIG. 2. Effect of 1-monoolein on aortic 2-monoacylglycerol (MG) lipase and diacylglycerol (DG) kinase activities. MG lipase activity (●) in the soluble aortic preparation (99 μg protein per assay; 100% = 301 Unit/mg protein) and DG kinase activity (△) in a microsomal preparation (60 μg protein per assay; 100% = 44.3 nmol phosphatidic acid formed/hr/mg protein) was measured in the presence of the indicated concentrations of 1-monoolein. A stock solution of 1-monoolein was prepared by sonication into 0.01% Triton X-100; activities are expressed as a percentage of control assays that contained the equivalent amount of detergent.

an oleate concentration of approximately 50 μM (panel A). The inhibition of 2-MG lipase activity due to the presence of 40 μM oleate could be prevented when albumin (0.05%, w/v) was also present in the assay (Fig. 5, panel B). Preincubation of the lipase preparation with oleate prior to the addition of the substrate mixture to initiate the assay produced no greater inhibition of lipase activity compared with the inhibition when the fatty acid was introduced directly to the assay incubation (results not shown). Albumin had no significant effect on lipase activity measured in the absence of oleate, presumably since the amount of oleate that would accumulate as the product under standard assay conditions (Figs. 1 and 4) would be less than 20 μM and thus would not appreciably inhibit lipase activity (Fig. 5).

2-MG lipase activity was inhibited by the addition of $CaCl_2$ to the assay (Fig. 6); 50% inhibition was observed at a concentration of 16 mM. Lipase activity was much less sensitive to inhibition by NaF where a concentration of 150 mM was necessary to produce 50% inhibition. By comparison, NaCl had no effect on lipase activity (Fig. 6).

Preincubation of the lipase preparation with p-bromophenacyl bromide (BPB) resulted in a concentration-dependent inhibition of 2-MG lipase activity (Fig. 7A). However, this inhibition could be prevented if DTT was also included in the preincubation (Fig. 7B). Preincubation with 500 μM phenylmethylsulfonyl fluoride (PMSF) for 20 min at room temperature also resulted in a reduction of lipase activity to 56% of control. 2-MG lipase activity was not significantly inhibited by the addition of 500 μM mepacrine or 200 μM indomethacin directly to the assay (results not shown).

DISCUSSION

The subcellular distribution of 2-MG lipase is tissue-specific, with activity reported to be either predominantly soluble in decidua vera (10) and adipose (15) tissues or mainly particulate in platelets (9). In the case of the aorta, 2-MG lipase activity was evenly distributed between soluble and particulate subcellular fractions (6), but a large fraction of the particulate activity could be solubilized with Triton X-100.

The apparent Km of 53 μM for the hydrolysis of 2-monoolein with the aortic lipase preparation is significantly less that other reported values ranging from 200 μM for the enzyme from adipose tissue (15) to 750 μM for the platelet 2-MG lipase (9); differences in the physical properties of the different substrate emulsions may contribute to these discrepancies. In previous work with a particulate fraction from rabbit aorta, an apparent Km of 83 μM was obtained for the release of the fatty acid from the *sn*-2 position of 1,2-DG (6). The soluble aortic lipase preparation demonstrated little or no positional substrate specificity when rates of hydrolysis were determined with 1- and 2-monoolein substrates (Fig. 1); similar results have been reported for MG lipases in platelet membranes (9) and for purified lipase preparations from adipose tissue (15) and intestinal mucosa (16).

This lack of positional specificity provides further evidence for the proposed two-step reaction sequence for the hydrolysis of 1,2-DG by aorta and microvessel preparations (6,7), since the accumulation of the 2-MG intermediate during incubations of 1,2-DG cannot be due to a preferential hydrolysis of 1-MG. The competitive inhibition of 2-MG lipase activity by 1-monoolein (Fig. 3) is evidence that the hydrolysis of both 1-monoolein and 2-monoolein is catalyzed by the same lipase. A concentration of approximately 200 μM 1-monoolein was required to produce a 50% inhibition of 2-MG lipase activity measured with 80 μM 2-monoolein as substrate (Fig. 2), even though rates of hydrolysis of both 1- and 2-monoolein were very similar when compared at a concentration of 80 μM (Fig. 1). This suggests that the affinity of the lipase for 1-monoolein is lower than for 2-monoolein, and indeed the K_i for 1-monoolein (65 μM) was greater than the Km of 53 μM for 2-monoolein (Fig. 3). The affinity and maximal velocity for the hydrolysis of 1-monoolein was not determined, but a lower affinity and higher maximal velocity, perhaps due to differences in the physical state of the substrate emulsion, could result in the same rate of hydrolysis at a single concentration as that observed with 2-monoolein as substrate.

The soluble aortic lipase preparation showed no prefer-

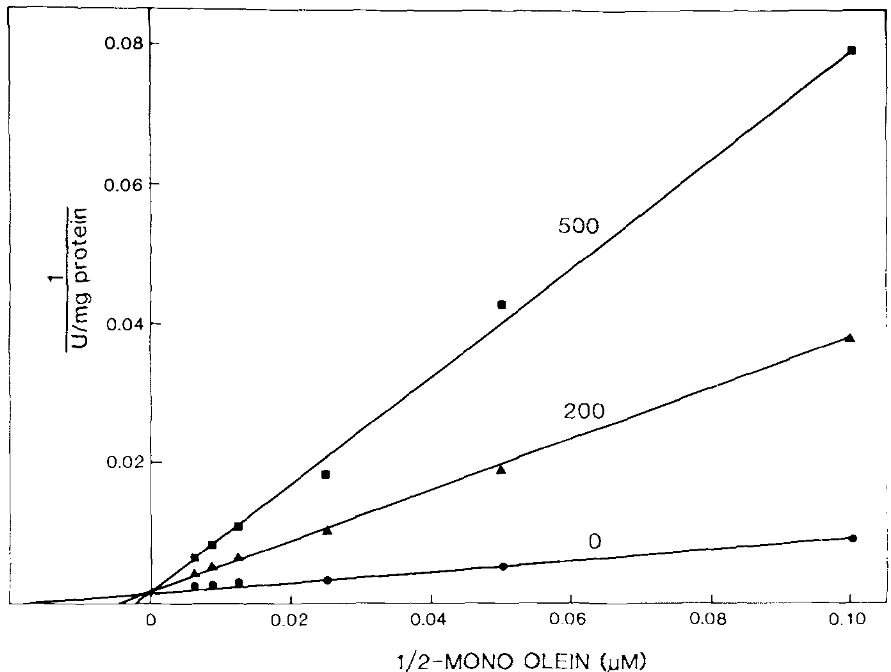

FIG. 3. Effect of 1-monoolein on 2-monoacylglycerol lipase activity. Lipase activity (99 μg protein per assay) was determined at varying 2-monoolein concentrations (10 to 160 μM) in the absence (●) and the presence of 200 μM (▲) and 500 μM (■) 1-monoolein. The respective 1-monoolein concentrations are indicated by the numbers on the lines. Results are presented as a double reciprocal Lineweaver-Burk plot.

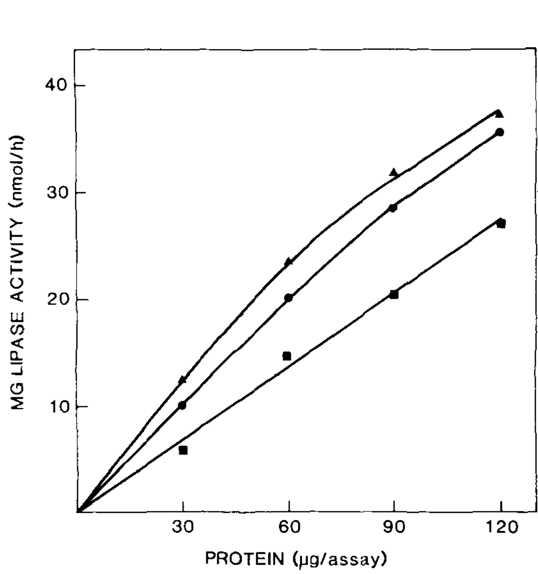

FIG. 4. 2-Monoacylglycerol (MG) substrate specificity for the aortic lipase. Lipase activity was measured after a 20 min incubation of the indicated amounts of enzyme protein with 2-monoolein (●), 2-monopalmitin (▲) and 2-monoarachidonin (■) substrate preparations.

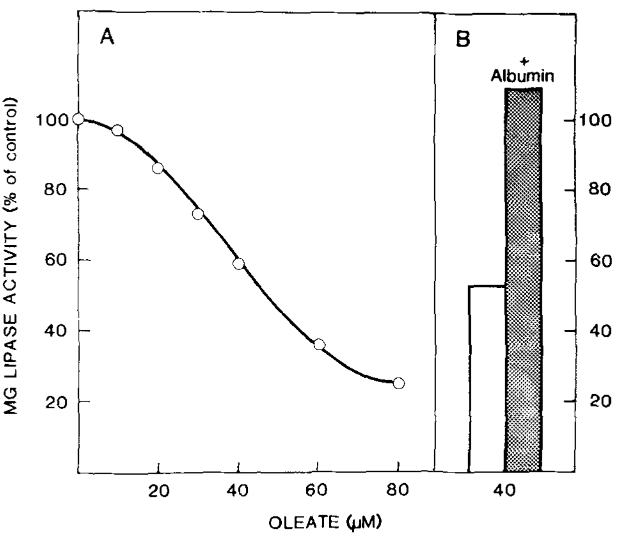

FIG. 5. Effect of oleate on 2-monoacylglycerol (MG) lipase activity. (A) Lipase activity was measured in the presence of the indicated concentrations of oleate. A 1-mM stock solution of sodium oleate was prepared in 10% methanol; control tubes contained an equivalent amount of methanol. Results are expressed as a percentage of the lipase activity determined in the absence of oleate (100% = 262 Units/mg protein). (B) Lipase activity was measured in assays with 40 μM oleate and in the absence (open bar) and in the presence (shaded bar) of albumin (0.05%, w/v). Activity is expressed as a percentage of the control activity with only methanol (100% = 232 Unit/mg protein).

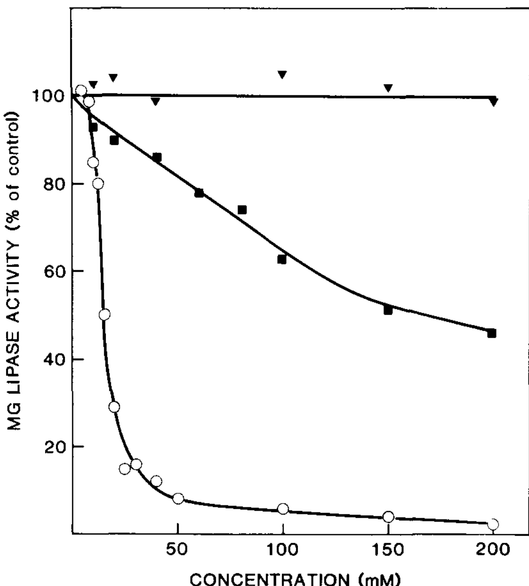

FIG. 6. Effect of NaCl, NaF and $CaCl_2$ on 2-monoacylglycerol (MG) lipase activity. Lipase activity was measured in the presence of the indicated concentrations of $CaCl_2$ (O), NaF (■) and NaCl (▼). Results are expressed as a percentage of control with no additions (100% = 268 Units/mg protein).

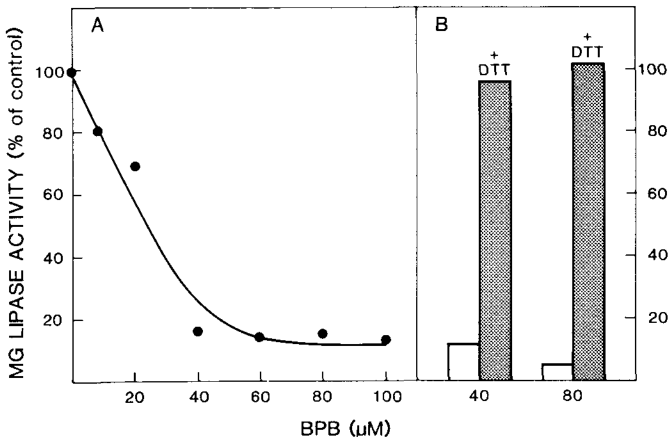

FIG. 7. Effect of p-bromophenacyl bromide (BPB) on 2-monoacylglycerol lipase activity. (A) A 10-mM BPB stock solution was freshly prepared in dimethyl sulfoxide (DMSO). The lipase preparation was preincubated for 20 min at room temperature in the presence of the indicated concentrations of BPB prior to initiation of the assay by the addition of the substrate mixture. Results are calculated as the percentage of control activity that was preincubated with an equivalent amount of DMSO (100% = 250 Units/mg protein). (B) Lipase activity was measured following preincubation with either 40 μM or 80 μM BPB and with no further additions (open bars) or in the presence of 1 mM dithiothreitol (DTT; shaded bars). Results are expressed as a percentage of the control (100% = 249 Units/mg protein).

ence for the hydrolysis of the naturally occurring substrate 2-monoarachidonin; in fact, the release of palmitate and oleate was greater than the release of arachidonate when the 2-MG substrates were compared (Fig. 4). This substrate specificity for the aortic 2-MG lipase is in marked contrast to the enzyme from platelets and decidua vera tissue (9,10), where the presence of arachidonate in the *sn*-2 position of 1,2-DG increased lipase activity. The inhibition of aortic 2-MG lipase activity by PMSF and NaF is consistent with results from other investigations (9,10,16). The aortic 2-MG lipase was also inhibited by oleate (Fig. 5). The ability of albumin to prevent and reverse the effect of added oleate indicates that the fatty acid acted by product inhibition and did not produce an irreversible denaturation of the lipase.

The DG lipase pathway in blood vessel preparations could provide the arachidonic acid precursor necessary for prostanoid synthesis. Alternatively, the arachidonic acid could be released by the direct action of a phospholipase A_2 on aortic membrane phospholipids (17). Recently, Moscat et al. (18) reported that phospholipase A_2 activity could not be detected in endothelial cell homogenates from pig aortas, and so concluded that the phosphoinositide-specific phospholipase C-DG lipase pathway must provide the arachidonic acid for prostanoid synthesis. Negative results for phospholipase A_2 activity measured in in vitro assays are always subject to qualifications concerning the appropriateness of the exogenous radiolabeled substrates (17). Furthermore, phospholipase A_2 activity has been characterized in homogenates and acid extracts of smooth muscle cells from aorta (19). Analysis of pig aortic endothelial cells following the stimulation of PGI_2 synthesis by bradykinin resulted in the conclusion that both phospholipase A_2 and phospholipase C-DG lipase pathways were involved in the supply of arachidonic acid (20).

The relative contributions of these two potential pathways in relation to the release of arachidonic acid for prostanoid synthesis in intact cells is difficult to evaluate without the availability of specific and selective inhibitors. BPB is a potent inhibitor of the aortic 2-MG lipase, presumably due to modifications of sulfhydryl groups, since the inhibition could be prevented by DTT (Fig. 7). However, BPB also inhibits phospholipase A_2 (19-22) and C (22,23) activities. Given this nonspecificity, BPB clearly cannot be used to determine the relative contributions of the two pathways to release arachidonic acid. Similarly, mepacrine (500 μM), although it did not inhibit aortic 2-MG lipase activity, has been reported to inhibit both phospholipase C (23) and A_2 (19) activities and has been demonstrated to interact directly with membrane phospholipids (24). Indomethacin resulted in a potentiation of the accumulation of DG in thrombin-stimulated platelets; indomethacin was reported to inhibit DG lipase activity but not DG kinase activity in platelet sonicates (25). In contrast, indomethacin (200 μM) did not significantly inhibit aortic 2-MG lipase activity. However, it is also well established that indomethacin is an effective inhibitor of phospholipase A_2 (21,26,27). Thus, all these inhibitors are nonspecific to varying degrees. Although RHC 80267 was developed as a selective DG lipase inhibitor (28), this compound has proven to be ineffective when incubated with intact platelets unless used at high concentrations that produce nonspecific inhibition of arachidonic acid release (29,30).

The inhibition of brain DG kinase by 1-monoolein was

first reported by Bishop et al. (14). Treatment of intact platelets with 1-monoolein resulted in a potentiation of the thrombin-induced rise in intracellular DG (14) and an attenuation of the metabolism of exogenous DG (31). Since 1-monoolein reduced the conversion of exogenous DG to water-soluble metabolites, it was suggested that the DG lipase pathway was inhibited along with DG kinase (31). Our results have shown that 1-monoolein is a potent inhibitor of both DG kinase and 2-MG lipase activities in aorta (Fig. 2). The Ki of 65 μM for the competitive inhibition of the 2-MG lipase by 1-monoolein can be compared to the Ki of 91 μM for the inhibition of brain DG kinase (14). If 1-monoolein does not inhibit phospholipase A_2 activity, then this compound could be a useful tool to investigate the contribution of the DG lipase pathway to arachidonic acid release from stimulated vascular endothelial and smooth muscle cells.

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L-Carnitine Effect on Plasma Lipoproteins of Hyperlipidemic Fat-Loaded Rats

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The effect of oral L-carnitine administration to rats fed olive oil has been studied. Carnitine significantly decreased triglyceride, cholesterol and phospholipid levels. Particularly, the levels of chylomicron and very low density lipoproteins in the blood were lowered. Low density lipoprotein levels were not affected, and high density lipoproteins were found to be decreased by 20%. Because carnitine did not change the composition of chylomicron and very low density lipoproteins fraction or affect the gastrointestinal triglyceride residue (about 1/3 of the original load), an effect of carnitine on hepatic fatty acid handling is most likely. The lowering of plasma free fatty acid levels by carnitine administration is in favor of an effect of carnitine on fatty acid handling. The effect on the liver is illustrated by the study of acetoacetate formation in in vitro perfused livers from previously olive oil loaded ± carnitine-treated rats. Carnitine pretreatment stimulated ketogenesis. It is speculated that carnitine administration, by promoting β-oxidation, lowers the production of very low density lipoproteins. This may be accomplished partly by an increase in the hepatic level of fatty acid binding protein, which also has been observed.
Lipids 22, 1005-1008 (1987).

L-Carnitine plays an essential role in the uptake of activated long-chain fatty acids into the mitochondria (1). Therefore, under carnitine-deficient conditions long-chain acyl-CoAs accumulate and predominantly participate in extra mitochondrial reactions such as triglycerides (TG) synthesis. Such an event occurs in lipid storage myopathies due to carnitine deficiency in muscles (2-4). In previous experiments, we demonstrated that L-carnitine administration is capable of reducing hyperlipidemia after oral oil bolus (5) and after fat diet feeding (6).

The electrophoretical pattern of plasma lipoproteins showed a decreased intensity of the pre-β band and the chylomicron band by L-carnitine treatment. This experiment was carried out to confirm the above findings through analyses performed with the density gradient ultracentrifugation method as described by Redgrave et al. (7) and to contribute to the clarification of the role played by L-carnitine in lipid metabolism.

EXPERIMENTAL MODEL

Three groups of normally fed, albino female Wistar rats, 200-220 g in weight, were treated as follows: control and L-carnitine groups were given olive oil (30 ml/kg) orally at time 0; one hr later, they received H_2O (20 ml/kg) and 155 mM L-carnitine (20 ml/kg), respectively. The blank group received H_2O (30 and 20 ml/kg) at the same times. At the third hour, blood samples were drawn under a light ether anesthesia for determination of free fatty acids (FFA) (8), TG (9), total cholesterol (CH) (10,11), phospholipids (PL) (12), total carnitine (TCAR) (13) and short-chain acylcarnitines (14) (the latter were obtained by subtracting the amount of free carnitine from total acid-soluble carnitine), protein (15) and lipoproteins (7). Under the same experimental conditions, some animals were used for liver short-chain acylcarnitines (14), intestinal TG determination and as donors of livers for (subsequent) in vitro perfusion studies (16), in which FFA (8) uptake and acetoacetate (17) production were measured. Fatty acid binding protein (FABP) was measured in the livers of the three groups of animals. This was accomplished by rocket electrophoresis of liver homogenates purified supernatant samples made in cold 0.2 M mannitol. The homogenates were centrifuged for 30 min at 48,000 × g at 4 C. The supernatants were heated for 10 min at 60 C and were cooled and centrifuged for 15 min at 48,000 × g. The supernatant was dialyzed against 75% saturated ammoniumsulfate overnight, and the precipitate was removed by centrifugation. The supernatant was dialyzed against water, freeze-dried and tested for FABP content by rocket electrophoresis (18). The 1% agarose used contained antiserum raised in rabbit against purified (19) rat liver FABP. The electrophoresis was run for 16 hr at 50 mV/4 cm.

Statistical analysis. Results were expressed as mean value + SEM. Student's t-test was used to evaluate the statistical significance of the differences.

RESULTS AND DISCUSSION

Plasma FFA, TG, CH and PL levels all are shown to have increased in the fat-loaded group (Table 1) as compared to the blank group. L-carnitine administered to the hyperlipidemic animals significantly reduced FFA (−65%; $P \leqslant .1\%$), TG (−44%; $P \leqslant 1\%$), CH (−35%; $P \leqslant .1\%$) and PL (−32%; $P \leqslant .1\%$), and it induced a dramatic increase in TCAR (six-fold; $P \leqslant .1\%$) (Table 1).

Plasma chylomicron (CHYLO) plus very low density lipoprotein (VLDL) and high density lipoprotein (HDL) fractions, which had raised significantly after the olive oil load, were lowered markedly by treatment with L-carnitine (−54%; $P \leqslant 2\%$, and −20%; $P \leqslant 5\%$, respectively) (Fig. 1). The low density lipoprotein (LDL) fraction in the plasma was affected moderately by the oil load and the successive L-carnitine treatment (Fig. 1).

All constituents of the CHYLO plus VLDL fraction were reduced almost equally (TG, −55%; CH, −59%; PL, −58%; protein, −61%) by the treatment with L-carnitine (Table 2). This suggests that carnitine modifies the quantity without affecting the composition of the CHYLO plus

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Abbreviations: CH, cholesterol; CHYLO, chylomicron; FABP, fatty acid binding protein; FFA, free fatty acids; HDL, high density lipoprotein; PL, phospholipids; TCAR, carnitine; TG, triglycerides; VLDL, very low density lipoprotein.

TABLE 1

Plasma Free Fatty Acids (μM), Triglycerides (mg/100 ml), Cholesterol (mg/100 ml), Phospholipids (mg/100 ml) and Total Carnitine (μM) in Blank, Control and L-carnitine Groups

	Animal group		
	Blank $H_2O + H_2O$	Control Olive oil + H_2O	L-carnitine Olive oil + L-carnitine
Free fatty acids	737 ± 89[a] (14)	1830 ± 251 (14)	629 ± 38[a] (14)
Triglycerides	89.7 ± 16[b] (8)	392 ± 59 (16)	220 ± 46[b] (19)
Cholesterol	44.9 ± 3.2[b] (8)	78.2 ± 8.6 (16)	50.9 ± 4.1[a] (19)
Phospholipids	133 ± 6.9[b] (8)	244 ± 24 (16)	165 ± 15[a] (19)
Total carnitine	56.9 ± 4.1[b] (8)	38.5 ± 1.9 (8)	244 ± 31[a] (11)

[a]P ⩽ .1% vs control.
[b]P ⩽ 1% vs control.
In parentheses, number of animals.

TABLE 2

Plasma Chylomicron (CHYLO) Plus Very Low Density Lipoprotein (VLDL) Triglycerides (mg/100 ml), Cholesterol (mg/100 ml), Phospholipids (mg/100 ml) and Protein (mg/100 ml) in Blank, Control and L-carnitine Groups

	Animal group		
	Blank $H_2O + H_2O$	Control Olive oil + H_2O	L-carnitine Olive oil + L-carnitine
CHYLO + VLDL − triglycerides	69.7 ± 15[a]	303 ± 75	136 ± 25[a]
Δ% vs Blank	—	+334	+95
Δ% vs Control	—	—	−55
CHYLO + VLDL − cholesterol	4.84 ± 0.9[b]	28.2 ± 6.1	11.6 ± 2.5[c]
Δ% vs Blank	—	+482	+139
Δ% vs Control	—	—	−59
CHYLO + VLDL − phospholipids	11.4 ± 3.0[b]	57.3 ± 13	23.8 ± 7.3[c]
Δ% vs Blank	—	+402	+108
Δ% vs Control	—	—	−58
CHYLO + VLDL − protein	4.2 ± 1.3	9.1 ± 1.9	3.0 ± 0.7[b]
Δ% vs Blank	—	+116	−29
Δ% vs Control	—	—	−67

[a]P ⩽ 2% vs control.
[b]P ⩽ 1% vs control.
[c]P ⩽ 5% vs control.
Number of animals = eight per group.

TABLE 3

Liver Fatty Acid Binding Protein Concentration (mg/g w.w. ± S.E.M.) in Blank, Control and L-carnitine Groups

Animal group		
Blank $H_2O + H_2O$	Control Olive oil + H_2O	L-carnitine Olive oil + L-carnitine
0.99 ± 0.11 (7)	1.10 ± 0.10 (8)	1.69 ± 0.25[a] (8)

[a]P ⩽ 5% vs control.
In parentheses, number of animals.

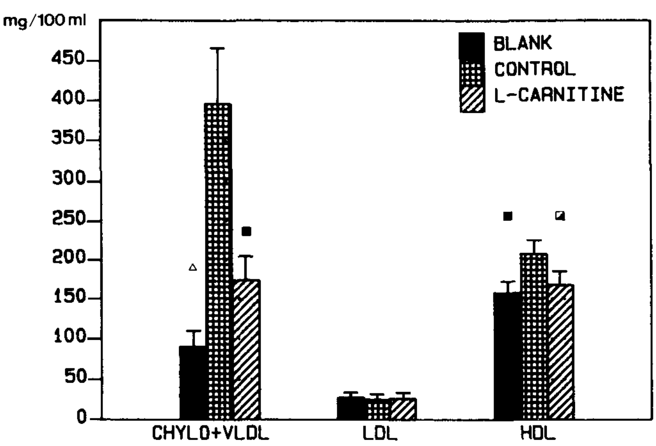

FIG. 1. Plasma chylomicron (CHYLO) plus very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoprotein (HDL) in blank (H_2O + H_2O), control (olive oil + H_2O) and L-carnitine (olive oil + L-carnitine) groups. Standard errors and P-values vs control (◪ ⩽ 5%; ■ ⩽ 2%; △ ⩽ 1%) are indicated in the figure. Number of animals: eight per group.

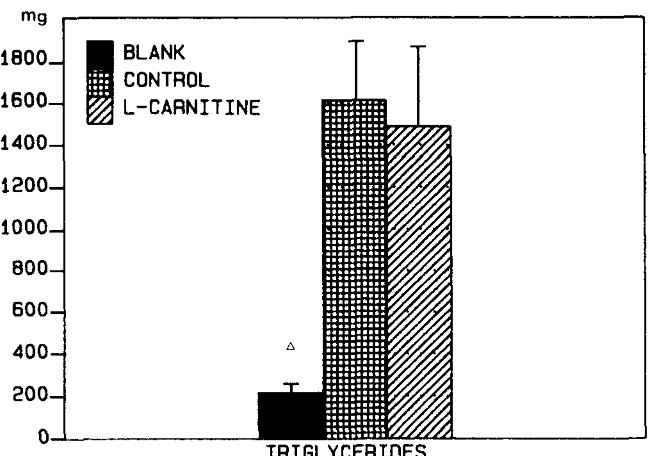

FIG. 3. Entire intestine triglycerides content (tissue + content) of blank (H_2O + H_2O), control (olive oil + H_2O) and L-carnitine (olive oil + L-carnitine) groups. Standard errors and P-values vs control (△ ⩽ 1%) are indicated in the figure. Number of animals: four per group.

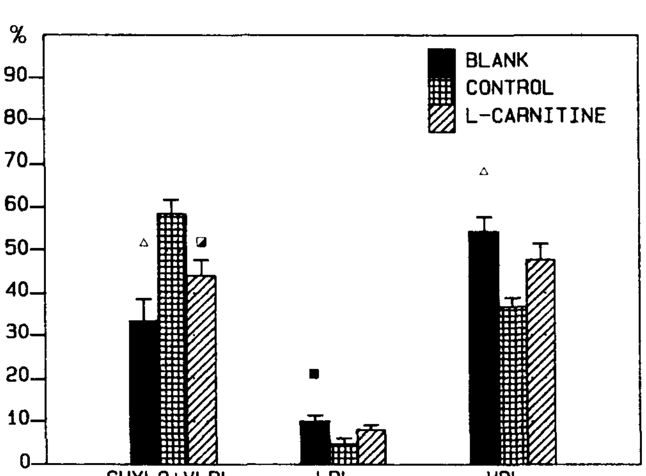

FIG. 2. Percent distribution of plasma lipoprotein components in blank (H_2O), control (olive oil + H_2O) and L-carnitine (olive oil + L-carnitine) groups. Standard erros and P-values vs control (◪ ⩽ 5%; ■ ⩽ 2%; △ ⩽ 1%) are indicated in the figure. Number of animals: eight per group.

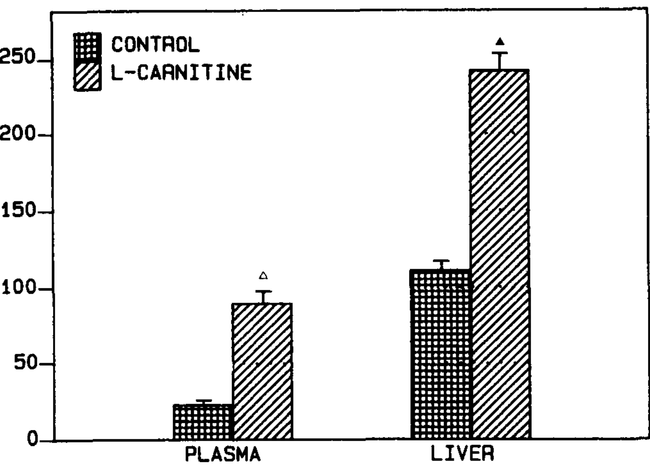

FIG. 4. Plasma (nmoles/ml) and liver (nmoles/g w.w.) short-chain acylcarnitines in control (olive oil + H_2O) and L-carnitine (olive oil + L-carnitine) groups. Standard errors and P-values vs control (△ ⩽ 1%; ▲ ⩽ .1%) are indicated in the figure. Number of animals: 14 per group.

VLDL fraction. As evidenced by previous experiments (5,6), the percentual distribution of plasma lipoprotein components of the L-carnitine group was different substantially from that of the control group (Fig. 2). The blank group, on the other hand, showed no statistical difference from the L-carnitine group (Fig. 2). As mentioned in *Experimental Model*, to ascertain that the effect of L-carnitine was not due to an interference with gastrointestinal lipid absorption, at the third hour some animals from the three groups were used to determine the levels of TG in the whole intestine (tissues and its contents). The same intestinal TG level, about 1/3 of original load, was found in both control and L-carnitine groups (Fig. 3).

Therefore, it is reasonable to assume that the effect of L-carnitine on TG-rich lipoproteins in the blood should not be ascribed to interference with gastrointestinal lipid absorption by the substance but rather to an influence it may exert on the handling of fatty acids by the liver. This hypothesis is in agreement with the following results: it can be seen from Figure 4 that in the L-carnitine group, plasma and hepatic levels of short-chain acylcarnitines were higher than the control group. In accordance with the results obtained by other authors with in vitro experiments (20), the livers of the animals treated with oil plus L-carnitine, isolated and perfused as described in

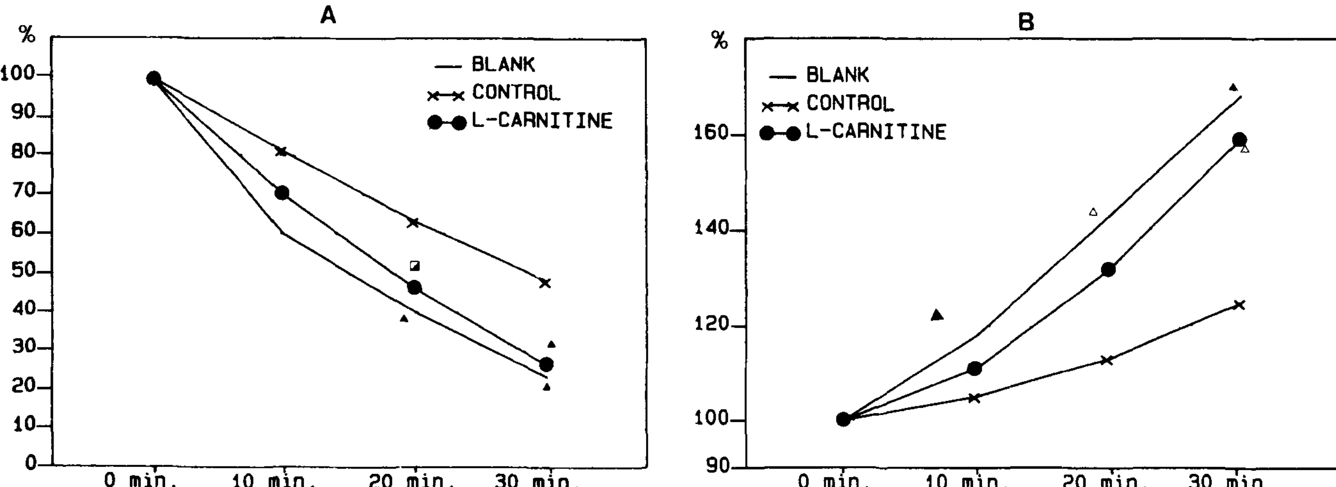

FIG. 5. Fatty acid uptake (side A) and acetoacetate release (side B) in isolated livers from rats previously loaded orally with olive oil and subsequently with carnitine as described in *Experimental Model* and in vitro perfused with recirculated medium (equilibrated with 95% O_2 and 5% CO_2 at 37 C). The medium (60 ml) contained 40% (v/v) serum and 15% (v/v) packed red cells of 48-hr fasted rats, supplemented with Krebs-Ringer bicarbonate buffer (pH 7.4), containing 11.5 mM glucose. The ordinate of side A, % of the initial FFA concentration (100% about 0.8 mM); side B, % of the increase in the acetoacetate level (100% about 0.5 mM). P-values (◪ ⩽ 5%; △ ⩽ 1%; ▲ ⩽ .1%) of blank (H_2O + H_2O) and L-carnitine (olive oil + L-carnitine) vs control (olive oil + H_2O) are indicated in the figure. Number of animals: three per group.

the legend of Figure 5, converted FFA into acetoacetate faster than the control liver did. Therefore, the data of Figures 4 and 5 suggest that carnitine addition after olive oil feeding promotes hepatic β-oxidation. This may be expected to occur at the expense of fatty acid esterification, leading to the lowering of circulating plasma fatty acyl esters.

Preliminary experiments suggest an increase of hepatic acid binding protein concentration (Table 3) by carnitine addition. Fatty acid binding protein promotes fatty acid utilization (21). Therefore, stimulation of fatty acid handling by the liver, promoting β-oxidation, is to be expected after carnitine administration.

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The Result of Feeding Palmitoyl Glycerol on Lymph and Plasma Lipids

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Palmitoyl glycerol is toxic when fed to mice, but the toxicity is alleviated by supplementing the toxic diet with 2–4% oleate or linoleate at the expense of sucrose. Lipid and fatty acid composition of lymph and plasma were studied in mice fed chow and palmitoyl glycerol diets to help explain the toxicity mechanism. When mice were fed chow, intestinal lymph contained a high proportion of saturated fatty acids; when they were given palmitoyl glycerol, the proportion approached 90% saturated fatty acids. The cholesteryl ester fraction was higher in lymph from mice fed a toxic diet than when the diet was fortified with supplemental safflower oil. However, there were no differences between diets in lipid composition of blood plasma. Similarly, except for plasma cholesterol esters, there were no differences in fatty acid composition between mice fed palmitoyl glycerol as the only fat or supplemented with a protective unsaturated fat. In the plasma, cholesteryl palmitate was elevated and cholesteryl oleate and cholesteryl linoleate were depressed when mice were given a toxic diet. Although a monoacylglycerol was toxic when fed, the percentages of monoacylglycerols in lymph or plasma were not materially elevated. The findings indicate that neither the total proportion of saturated fatty acids nor the amount of circulating monoacylglycerols was directly involved in the toxicity of palmitoyl glycerol.
Lipids 22, **1009–1015 (1987).**

Rac-1(3)-palmitoyl glycerol was toxic when fed to weanling mice at dietary percentages between 10 and 30% (1). Adding 2% oleate or linoleate (safflower oil) at the expense of sucrose to the *rac*-1(3)-palmitoyl glycerol diet greatly ameliorated the toxicity (1). The symptoms of the toxicity were death, usually within seven days after starting the diet, and poor growth in those mice that survived (2). The toxicity was not as severe in adult mice or weanling rats as in weanling mice (2). There was a pronounced effect of ambient temperature on the toxicity of the palmitoyl glycerol diet (3). Death did not occur when the ambient temperature was greater than 25 C. Moreover, at ambient temperatures below 25 C, the mortality was inversely proportional to the temperature. Although a severe inflammation of the lungs was observed in about 75% of the mice that died, no lung abnormality was observed in the other 25% that died (2). Moreover, the lung lesion was as severe in those mice that survived because they were kept at an ambient temperature above 25 C as in those that died when held at the lower ambient temperature (3).

For these reasons, it is clear that even though the inflammatory lesion of the lung is a symptom of the toxicity, it is not the immediate cause of death. There was no difference between mice fed *rac*-1(3)-palmitoyl glycerol with or without safflower oil in the amount or rate of *rac*-1(3)-palmitoyl glycerol absorbed, oxidative phosphorylation by liver or heart mitochondria or tissue distribution of radioactivity following gavage of *rac*-1(3)-[1-^{14}C]-palmitoyl glycerol (2). The mechanism by which *rac*-1(3)-palmitoyl glycerol induces the toxic symptoms is unknown. Since the *rac*-1(3)-palmitoyl glycerol is fed and is absorbed, it seemed logical to ascertain if the lymph and/or plasma lipids from mice fed the toxic diet might be different from those fed the toxic diet protected by supplementation of an unsaturated fat. Such differences might lead to an understanding of the mechanism behind the toxicity and, more specifically, reveal whether the monoacylglycerol per se is the toxic compound after absorption. Therefore, studies on the fatty acid composition of the lymph and plasma lipids from mice fed varying proportions of *rac*-1(3)-palmitoyl glycerol, with or without dietary supplements of safflower oil or oleic acid, were conducted. The results of these studies are reported in this communication.

MATERIALS AND METHODS

Animals. The animals used in these experiments were weanling and adult mice from the North Carolina State Laboratory of Hygiene (strain: NC St Lab of Hygiene), weanling mice from Charles River (strain: CFW) and adult rats (strain: Charles River Wistar). Usually the mice were fed a basal diet containing 40% sucrose, 20% casein and 5% Wesson salt mixture and supplemented with all known vitamins (ICN Pharmaceuticals, Cleveland, OH). The remaining 34% was composed of *rac*-1(3)-palmitoyl glycerol and safflower oil, oleic acid and sucrose. Some mice and rats were fed an open-formula, commericial rodent diet (chow) to serve as control animals. This contained about 7% fat, with an unsaturated fatty acid composition of 35% oleate and 25% linoleate. Additionally, some weanling mice were fed the same purified basal diet, with all fat replaced by sucrose. Mice were housed individually in galvanized cages with wire-screen bottoms at an ambient temperature of 19–22 C. Food and tap water were provided ad libitum. The *rac*-1(3)-palmitoyl glycerol was prepared as previously described (1) from acetone glycerol (Aldrich Chemical Co., Milwaukee, WI) and palmitic acid and was crystallized from hexane to a purity between 85 and 95%, with free palmitic acid as the major impurity and dipalmitoyl glycerol and tripalmitoyl glycerol as minor impurities. The previous study (1) established the veracity of *rac*-1(3)-palmitoyl glycerol as the dietary toxic compound.

Collection of lymph and plasma. Weanling mice were killed three days, and adult mice seven days, after starting the experimental treatments. Immediately after the mice were killed by cervical dislocation, intestinal lymph was collected by puncture of the mesenteric lymph duct with a tuberculin syringe equipped with a 26-gauge needle. The lymph was withdrawn into the syringe and flushed with 0.2 ml of chloroform/methanol (1:2, v/v) to extract the lipids (4). In this manner, we obtained about 1–3 μl from each animal, and it was necessary to pool the

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lipid extracts from six weanling mice or two adult mice to obtain amounts sufficient for the analyses. Because of the small size of the sample, we made no attempt to isolate the chylomicrons. Lymph from each rat was collected and extracted in the same manner.

Blood was collected from the mice by retro orbital puncture using a preheparinized Pasteur pipette. Plasma was collected after centrifugation at 1000 × g for 10 min at 4 C. The plasma was added to six volumes of chloroform/methanol (2:1, v/v), and the lipids were extracted (4).

Lymph and plasma lipid and fatty acid composition. The extracts of the lymph and plasma were taken to dryness by a stream of nitrogen and redissolved in a small volume of chloroform. The lipid classes were separated by thin layer chromatography on Silica Gel H (American Scientific Products, Charlotte, NC) using a solvent mixture of heptane/isopropyl ether/acetic acid (6:4:0.3, v/v/v). The diacylglycerol, triacylglycerol, free fatty acid and cholesteryl ester regions were collected after visualization by a brief exposure to iodine vapor, and lipids were eluted with chloroform/methanol (1:1, v/v). The fraction containing both the phospholipids and monoacylglycerols was eluted from the silica gel with ethanol/chloroform/water/acetic acid (100:30:20:2, v/v/v/v). Then the phospholipid and monoacylglycerol fractions were separated by rechromatography on thin layer plates using a solvent system of acetone/petroleum ether (1:3, v/v) (5). The phospholipids were eluted with the same solvent mixture previously used, and the monoacylglycerols were eluted with a mixture of chloroform/ether (1:1, v/v). All lipid fractions were taken to dryness with a stream of nitrogen, and 10 μg of heptadecanoic acid (NuChek Prep, Elysian, MN) was added as an internal standard in chloroform. Methyl esters of the fatty acids of each lipid class were prepared by transmethylation with a mixture of 2 ml of methanol, 0.2 ml of benzene, 0.1 ml of concentrated HCl and 0.1 ml of dimethoxypropane to serve as a water scavenger. The reaction mixture was kept at 65 C for 30 min and allowed to stand overnight at room temperature. The fatty acid methyl esters were collected by adding 2 ml of water and extracting three times each with 2 ml of petroleum ether. After being brought to dryness with a stream of nitrogen, the fatty acid methyl esters were dissolved in a small volume of ether, and the composition was determined by gas-liquid chromatography on a 1/8-inch stainless steel column packed with 10% diethyleneglycol succinate (Supelco, Bellefonte, PA).

To insure the accuracy of basing lipid analysis on the amounts of fatty acids in the lipid fractions eluted from the thin layer plate, recovery experiments using pure known standards were conducted. In these experiments, known amounts of tripalmitoyl glycerol, dipalmitoyl glycerol, palmitoyl glycerol, cholesteryl oleate, phosphatidylcholine and phosphatidylinositol were spotted on a thin layer plate. These samples were then handled as the experimental samples, with recovery based on the ratio of the amounts of fatty acid(s) to the internal standard of heptadecanoic acid. The recoveries of the pure lipids were, respectively, 96.4%, 95.2%, 98.5%, 90.0%, 91.6% and 98.0%.

Results are expressed as means and standard deviations of the means. Student's t-test was used to determine statistical significance.

RESULTS

Lipid composition of lymph. The lipid composition of rat lymph was similar to that previously reported (6) in that the major lipid was the triacylglycerol fraction and the sum of the remaining fractions constituted only 35% of the total lipids (Table 1). Even when they were fed laboratory chow, the lipid composition of lymph from both weanling and adult mice differed from that of adult rats in that the triacylglycerol fraction, although still the major one, amounted to less than half of the total lipids. This decrease was compensated by higher proportions of cholesteryl esters and free fatty acids.

The triacylglycerol fraction was even lower in lymph from weanling mice fed *rac*-1(3)-palmitoyl glycerol supplemented with safflower oil, and it was lowest in lymph from either weanling or adult mice fed the toxic palmitoyl glycerol diet. The cholesteryl ester fraction was the major lipid in the lymph from both groups of mice fed *rac*-1(3)-palmitoyl glycerol without an unsaturated fat. However,

TABLE 1

Lipid Composition of Lymph[a]

Diet	Animal	N	MG (%)	DG (%)	TG (%)	CE (%)	FFA (%)	PL (%)
Chow	Adult rat	2	11.5	3.9	64.9	8.1	9.2	2.5
Chow	Weanling mice	5	10.4(4.7)	5.5(5.7)	45.6(8.5)	17.9(0.9)	14.5(5.7)	5.8(2.5)
Chow	Adult mice	4	11.7(7.3)	8.7(2.0)	41.3(7.1)	15.7(3.3)	16.1(1.0)	6.2(3.6)
GMP	Weanling mice	4	15.5(3.1)	12.3(3.9)	15.6(2.5)[b]	30.4(5.1)[b]	20.9(6.7)	5.4(1.6)
GMP + SO	Weanling mice	4	14.7(1.7)	11.0(4.3)	27.5(3.6)[b,c]	15.5(3.1)[c]	27.5(3.6)[b]	4.4(2.4)
GMP	Adult mice	5	7.4(1.2)	13.6(3.5)	23.0(5.8)[b]	27.3(6.2)[b]	23.9(4.0)	3.9(2.5)
GMP + SO	Adult mice	5	8.7(2.1)	7.9(1.9)	60.3(6.2)	10.5(3.3)	10.3(2.2)	2.3(0.5)

[a]Values are means with standard deviations in parentheses; N, number of samples analyzed. Each sample represents a pool of lymph from 6 mice. Intestinal lymph from rats and mice fed the indicated diets was collected by aspiration from the mesenteric lymph node. The lipids were extracted and separated by thin layer chromatography. Quantitation was based on fatty acid analysis with recovery monitored by means of a heptadecanoic acid internal standard. MG, monoacylglycerols; DG, diacylglycerols; TG, triacylglycerols; CE, cholesteryl esters; FFA, free fatty acids; PL, phospholipids; GMP, *rac*-1(3)-palmitoyl glycerol; SO, safflower oil.

[b]Statistically different from chow ($p < 0.01$).

[c]Statistically different from GMP ($p < 0.01$).

when weanling mice were fed the safflower oil supplement, the cholesteryl ester fraction was not higher than the chow-fed mice, and the free fatty acids were increased. In contrast, the lymph triacylglycerol and cholesteryl ester percentages from adult mice fed *rac*-1(3)-palmitoyl glycerol and safflower oil were equivalent to that of adult rats fed chow.

It is significant that despite a dietary level of 30% palmitoyl glycerol, the proportion of monoacylglycerols in lymph from all groups of mice was similar and no higher than that of the adult rat.

Lipid composition of plasma. The composition of lipids in the plasma from weanling mice fed laboratory chow, a fat-free diet and 30% *rac*-1(3)-palmitoyl glycerol with and without 4% safflower oil are shown in Table 2. As might be expected, the percentage of triacylglycerols in the plasma of mice fed the fat-free diet was low, and the cholesteryl ester fraction was the major component. In contrast to lymph, the plasma triacylglycerols were higher and cholesteryl esters were lower when mice were fed palmitoyl glycerol with or without safflower oil as compared to those fed chow.

Fatty acid composition of lymph. The fatty acid composition of the lipids of the lymph from adult mice is shown in Table 3 and that from weanling mice in Table 4. With either weanling or adult mice fed chow, the triacylglycerol fatty acids reflected the fatty acid composition of the diet. Similarly, when either age group was fed a palmitoyl glycerol diet, a higher percentage of palmitate was found than when the mice were fed chow. With adult mice, supplementing the palmitoyl glycerol diet with safflower oil elevated the triacylglycerol linoleate, and in weanling animals a similar change was seen, but to a lesser degree. It is interesting that the percentage of palmitate in the lymph triacylglycerols of the mice receiving the safflower oil supplement was not significantly lower than those consuming the palmitoyl glycerol diet without the unsaturated fat.

In either adult or weanling mice, a palmitoyl glycerol diet resulted in higher palmitate in other lymph lipids compared to a chow diet. As with the triacylglycerols, adding a supplement of safflower oil did not affect these proportions significantly. All of the lipid classes of the lymph from weanling mice fed chow contained elevated percentages of stearate that offset the high palmitate percentage. Hence, the proportions of total saturated fatty acids in these lymph lipids from weanling mice were similar whether they were fed a palmitoyl glycerol diet or chow. Furthermore, in adult mice fed the palmitoyl glycerol diets, the linoleate percentages of the phospholipid fraction were considerably higher when the diet was supplemented with safflower oil.

Because the lymph triacylglycerol fatty acids were not markedly affected by safflower oil supplementation of the 30% palmitoyl glycerol diet, the fatty acids of this fraction from mice fed varying percentages of palmitoyl glycerol (ranging from 0 to 30%) and safflower oil (ranging from 0 to 4%) were analyzed. There was a linear increase in the proportion of linoleate (coefficient of correlation = 0.918) when the ratio of dietary safflower oil to palmitoyl glycerol was increased (Fig. 1). In contrast, the proportion of palmitate was not affected by increasing the dietary safflower oil-to-palmitoyl glycerol ratio.

Fatty acid composition of plasma. The proportions of the major fatty acids in the glycerolipids and free fatty acids of plasma from weanling mice fed chow, 30% palmitoyl glycerol as the only lipid, supplemented with either 4% safflower oil or 4% oleic acid, and a fat-free diet are given in Table 5. With the exception of the fat-free diet, the fatty acid compositions of the neutral lipids and free fatty acids from plasma of all diets are surprisingly similar, especially when one considers the wide variation in fat intake. It is only in the phospholipid fraction that consistent differences reflecting the fatty acid composition of the diet were observed. In contrasting the fat-free diet with the others, the most striking difference was its relatively high proportion of myristate.

In contrast, significant differences in fatty acid composition associated with diet were observed in the plasma cholesteryl ester fraction (Table 6). The palmitate percentage in this fraction was considerably higher when mice were fed palmitoyl glycerol alone rather than with either oleate or linoleate. When supplemented with 4% safflower oil, the proportion of linoleate in the cholesteryl ester fraction was 10-fold greater than when just palmitoyl glycerol was fed, and it was equal to that of the chow-fed mice.

TABLE 2

Lipid Composition of Blood Plasma from Weanling Mice[a]

Diet	N	MG (%)	DG (%)	TG (%)	CE (%)	FFA (%)	PL (%)
Chow	4	5.2(0.5)	9.2(0.9)	12.5(7.8)	42.8(9.6)	12.1(3.1)	15.2(2.8)
Fat-free	9	4.5(2.2)	3.3(1.8)[b]	6.6(5.7)	45.8(16.4)	8.2(2.8)[c]	31.5(11.9)[c]
GMP	6	9.0(2.1)[b,d]	6.9(1.7[c,d]	29.8(6.8)[b,d]	27.5(6.8)[c,e]	9.5(3.0)	18.0(7.1)[e]
GMP + SO	6	8.1(1.4)[b,d]	9.3(3.3)[d]	22.7(5.9)[c,d]	28.6(5.9)[c,e]	7.6(2.2)[c]	23.6(2.7)[b]

[a]Values are means with standard deviations in parentheses. N, number of mice. Blood plasma from weanling mice fed the indicated diets was collected by retro orbital puncture, and the plasma lipids were extracted and separated by thin layer chromatography. Quantitation was based on fatty acid analysis with recovery monitored by means of a heptadecanoic acid internal standard. MG, monoacylglycerols; DG, diacylglycerols; TG, triacylglycerols; CE, cholesteryl esters; FFA, free fatty acids; PL, phospholipids; GMP, *rac*-1(3)-palmitoyl glycerol; SO, safflower oil.

[b]Statistically different from chow ($p < 0.01$).

[c]Statistically different from GMP ($p < 0.01$).

[d]Statistically different from fat-free ($p < 0.01$).

[e]Statistically different from fat-free ($p < 0.05$).

TABLE 3

Fatty Acid Composition of Lymph Lipids of Adult Mice[a]

Diet	Lipid	Palmitate (%)	Stearate (%)	Oleate (%)	Linoleate (%)	Saturated (%)
Chow	CE	52.5(10.7)[g]	11.1(3.9)[f]	22.2(6.9)[f]	4.1(0.6)[g]	72.2(6.7)
GMP	CE	83.1(3.6)[b,e,g]	0.7(0.4)[b,f]	9.3(3.9)[e]	0.7(0.3)[b]	90.2(3.6)[b,e]
GMP + SO	CE	71.4(8.5)[b]	1.0(0.1)[f]	14.7(2.4)	2.2(1.6)	81.0(5.3)
Chow	DG	53.6(8.6)	6.7(1.5)	27.6(6.5)	4.1(2.2)	65.4(6.3)
GMP	DG	77.0(5.4)[b]	1.3(0.6)[b,f]	8.2(2.5)	3.0(1.7)	88.8(3.6)[b]
GMP + SO	DG	76.6(6.4)	1.3(0.7)[f]	11.3(4.5)	2.7(2.4)	85.9(3.8)[b]
Chow	FFA	50.1(6.2)	15.8(5.9)[f]	25.3(3.5)[f]	5.3(1.2)	69.3(2.1)[f]
GMP	FFA	77.6(4.5)[b,g]	1.0(0.1)[b,f]	11.4(6.9)[b]	1.4(1.1)[b]	87.1(6.6)[b]
GMP + SO	FFA	70.8(4.7)[b,e]	1.2(0.5)[b,f]	14.2(4.5)[b]	2.3(0.4)[b]	83.3(4.9)[b]
Chow	MG	43.9(3.9)[g]	7.5(7.1)[f]	40.5(5.4)[f]	2.1(0.6)[f]	57.3(5.3)[f]
GMP	MG	67.2(3.8)[b]	1.2(0.4)[f]	14.4(3.2)[b]	1.7(1.3)	85.1(5.1)[b]
GMP + SO	MG	65.9(12.6)	1.2(0.5)[f]	12.8(6.3)	0.9(0.2)[f]	84.8(8.9)[b]
Chow	PL	42.0(4.7)	9.0(4.9)[f]	31.1(7.4)[f]	7.7(6.3)	58.7(4.2)[f]
GMP	PL	55.1(11.3)	1.0(0.1)[b,f]	20.9(4.1)	1.3(0.8)[f]	76.3(4.9)[b]
GMP + SO	PL	55.6(8.0)[c]	4.9(1.3)[d,g]	21.3(6.4)	9.1(5.3)[e]	69.6(5.9)[c]
Chow	TG	30.4(2.6)	7.9(2.3)[f]	33.6(1.4)[f]	21.0(0.2)	41.1(3.9)
GMP	TG	81.5(5.3)[b,e]	1.9(1.3)[b,f]	9.4(3.6)[b]	1.8(1.0)[b,d]	88.8(4.2)[b,d]
GMP + SO	TG	72.9(4.5)[b]	1.8(0.3)	6.6(0.6)[b]	15.5(4.9)[f]	77.5(4.8)[b]

[a]Values are means, with standard deviations in parentheses, of fatty acid composition of the lipids of adult mice shown in Table 1. GMP, *rac*-1(3)-palmitoyl glycerol; SO, safflower oil; CE, cholesteryl ester; DG, diacylglycerols; FFA, free fatty acids; MG, monoacylglycerols; PL, phospholipids; TG, triacylglycerols.
[b]Statistically different from chow ($p < 0.01$).
[c]Statistically different from chow ($p < 0.05$).
[d]Statistically different from GMP + SO ($p < 0.01$).
[e]Statistically different from GMP + SO ($p < 0.05$).
[f]Statistically different from weanling (Table 4) ($p < 0.01$).
[g]Statistically different from weanling (Table 4) ($p < 0.05$).

TABLE 4

Fatty Acid Composition of Lymph Lipids of Weanling Mice[a]

Diet	Lipid	Palmitate (%)	Stearate (%)	Oleate (%)	Linoleate (%)	Saturated (%)
Chow	CE	42.9(4.9)[g]	27.5(2.5)[f]	5.7(3.6)[f]	2.9(0.7)[g]	74.9(7.9)
GMP	CE	73.1(8.2)[b,g]	8.3(1.9)[b,f]	12.9(7.2)	1.2(0.7)	84.4(5.7)
GMP + SO	CE	74.3(10.4)[b]	7.6(2.5)[b,f]	10.5(4.5)	1.7(0.1)	86.8(10.4)
Chow	DG	49.4(3.9)	23.6(5.2)[f]	11.2(4.5)[f]	3.5(1.4)	85.8(8.7)[f]
GMP	DG	68.5(10.5)[c]	8.7(1.7)[b,f]	16.8(7.6)[g]	2.3(1.8)	79.1(9.2)
GMP + SO	DG	69.4(4.9)[b]	7.3(1.3)[b,f]	13.1(2.6)	2.1(0.4)	82.7(2.8)
Chow	FFA	41.5(5.3)	36.2(7.6)[f]	11.3(4.3)[f]	5.0(2.5)	83.2(7.3)[f]
GMP	FFA	67.2(6.8)[b,g]	12.2(3.4)[b,f]	14.6(2.7)	1.4(0.2)[c]	82.9(3.2)
GMP + SO	FFA	70.5(8.5)[b]	8.5(0.1)[b,f]	13.6(2.4)	2.9(1.6)	83.3(5.3)
Chow	MG	55.1(7.1)[g]	25.7(2.6)[f]	10.3(4.6)[f]	<0.1[f]	90.0(7.5)[f]
GMP	MG	67.6(6.1)[c]	6.3(0.9)[b,f]	13.1(3.4)	3.1(0.8)[b]	78.7(4.3)[c]
GMP + SO	MG	71.2(9.4)[b]	6.1(1.5)[b,f]	8.9(3.8)	4.2(0.6)[b,f]	84.1(7.6)
Chow	PL	47.6(5.1)	28.1(9.3)[b,f]	9.7(3.6)[f]	5.4(1.4)	84.8(5.4)[f]
GMP	PL	58.3(3.7)[c]	9.1(1.2)[b,f]	17.6(2.3)[b]	3.8(0.7)[f]	72.6(2.6)[b]
GMP + SO	PL	55.8(5.4)	7.6(1.4)[g]	16.5(4.6)	5.0(1.7)	71.9(5.5)[c]
Chow	TG	32.3(4.9)	13.7(1.0)[f]	23.6(5.1)[f]	18.9(4.5)	50.5(8.9)
GMP	TG	73.4(9.2)[b]	7.6(1.3)[b,f]	12.6(7.6)	1.7(1.1)[b]	85.5(8.4)[b]
GMP + SO	TG	70.2(4.9)[b]	3.7(0.9)[b,d,f]	9.5(2.9)[b]	5.7(1.3)[b,d]	80.2(4.5)[b]

[a]Values are means, with standard deviations in parentheses, of fatty acid composition of the lipids of weanling mice shown in Table 1. GMP, *rac*-1(3)-palmitoyl glycerol; SO, safflower oil; CE, cholesteryl ester; DG, diacylglycerols; FFA, free fatty acids; MG, monoacylglycerols; PL, phospholipids; TG, triacylglycerols.
[b]Statistically different from chow ($p < 0.01$).
[c]Statistically different from chow ($p < 0.05$).
[d]Statistically different from GMP + SO ($p < 0.01$).
[e]Statistically different from GMP + SO ($p < 0.05$).
[f]Statistically different from adult (Table 3) ($p < 0.01$).
[g]Statistically different from adult (Table 3) ($p < 0.05$).

TABLE 5

Fatty Acid Composition of Glycerolipids from Plasma of Weanling Mice[a]

Diet	Lipid	Myristate (%)	Palmitate (%)	Stearate (%)	Oleate (%)	Linoleate (%)	Saturated (%)
Chow	DG	1.0(0.5)	74.0(0.5)	5.9(1.2)	16.3(1.2)	3.1(0.8)	81.3(1.7)
GMP	DG	5.4(1.5)[b]	62.5(10.3)	12.6(4.1)[c]	14.9(7.8)	1.3(1.2)[c]	80.7(10.7)
GMP + SO	DG	8.6(6.4)[c]	54.3(8.5)[b]	11.9(5.9)	16.8(2.6)	3.9(2.6)	75.8(5.4)
GMP + Oleate	DG	9.1(6.6)[c]	60.6(5.2)[b]	10.5(3.5)[c]	16.1(4.5)	<1	80.2(5.7)
Fat-free	DG	26.8(9.2)[e,f]	23.6(6.2)[e,f]	7.4(2.9)[d]	40.7(8.1)[e,f]	1.2(1.1)[c,g]	57.8(8.4)
Chow	FFA	4.4(3.5)	74.7(5.8)	6.6(1.0)	13.9(4.2)	1.5(0.9)	85.5(6.2)
GMP	FFA	5.7(2.0)	58.3(10.0)[c]	14.5(6.2)	18.2(8.4)	2.3(1.4)	78.4(10.6)
GMP + SO	FFA	5.8(4.0)	52.9(7.8)[b]	12.8(3.5)	22.2(13.1)	3.5(2.2)	70.4(14.6)
GMP + Oleate	FFA	6.5(4.6)	62.9(8.3)	12.2(2.3)	14.9(5.0)	1.7(1.0)	82.0(8.9)
Fat-free	FFA	18.1(4.9)[b,f]	40.5(4.1)[b,f]	21.2(4.6)	18.5(4.9)	1.2(1.2)	80.4(5.3)
Chow	MG	0.6(0.4)	43.2(7.9)	2.4(1.1)	48.0(7.5)	5.0(3.1)	48.3(8.9)
GMP	MG	8.2(6.7)	57.8(4.9)[c]	14.7(3.9)[c]	15.2(6.1)	2.9(1.4)	80.8(8.8)
GMP + SO	MG	10.8(5.5)	55.4(7.9)[b]	12.3(2.8)[b]	15.4(4.1)	1.1(0.5)	78.5(6.8)
GMP + Oleate	MG	5.8(2.8)	66.2(7.6)[b]	10.2(1.8)[b]	11.5(5.2)[b]	2.7(1.0)	82.2(9.2)
Fat-free	MG	31.1(8.7)[b,f]	29.8(2.9)[b,f]	11.5(2.7)[b]	25.5(7.0)[b,e,f]	1.6(1.3)[c]	72.4(6.8)
Chow	PL	2.5(1.6)	39.5(2.9)	22.6(0.6)	11.8(1.6)	23.6(2.3)	64.6(4.5)
GMP	PL	<1	54.5(3.9)[b]	24.1(10.9)	7.7(2.9)[c]	13.6(3.2)[b]	78.5(1.3)[b]
GMP + SO	PL	<1	45.1(2.7)[c,d]	25.9(2.9)	5.8(1.2)[b]	21.6(3.8)[d]	70.7(5.0)[d]
GMP + Oleate	PL	<1	55.8(5.8)[b]	27.2(0.9)[b]	14.7(5.4)[e]	3.4(1.3)[b,d]	81.0(6.5)[b]
Fat-free	PL	4.1(4.2)	38.9(6.3)[f]	18.6(6.5)[f]	23.6(8.8)[c,f]	8.9(5.0)[b,f]	61.6(13.5)
Chow	TG	3.2(1.7)	51.9(7.8)	5.7(1.8)	23.6(5.6)	14.4(3.1)	60.8(9.5)
GMP	TG	8.9(6.4)	54.1(14.3)	12.0(3.5?)[c]	20.6(13.5)	2.9(2.6)[b]	74.9(18.2)
GMP + SO	TG	4.6(3.5)	67.8(3.9)[b]	8.7(3.8)	11.5(2.6)[b]	5.1(3.8)[b]	81.0(5.1)[b]
GMP + 18:1	TG	3.3(1.9)	60.4(2.8)	4.8(1.3)[d]	26.9(5.9)	1.0(0.3)[b]	68.5(4.7)
Fat-free	TG	13.2(7.7)[c,g]	39.5(5.4)[b,e,f]	16.9(9.3)[c,g]	27.1(13.4)[g]	1.5(1.6)[b,g]	68.0(17.1)

[a]Values are means with standard deviations in parentheses. MG, monoacylglycerols; DG, diacylglycerols; TG, triacylglycerols; FFA, free fatty acids; PL, phospholipids. Number of mice and diets: GMP, 6 mice fed 30% palmitoyl glycerol; GMP + SO, 6 mice fed 30% palmitoyl glycerol + 4% safflower oil; GMP + Oleate, 4 mice fed 30% palmitoyl glycerol + 4% oleic acid; chow, 4 mice fed standard commercial pelleted diet; fat-free, 9 mice fed the GMP diet with palmitoyl glycerol replaced by sucrose. Blood plasma was extracted and the lipids were separated by thin layer chromatography. Fatty acid composition was determined by gas-liquid chromatography of the methyl esters. Only those fatty acids present in more than trace amounts are shown.

[b]Statistically different from chow ($p < 0.01$).

[c]Statistically different from chow ($p < 0.05$).

[d]Statistically different from GMP ($p < 0.01$).

[e]Statistically different from GMP ($p < 0.05$).

[f]Statistically different from fat-free ($p < 0.01$).

[g]Statistically different from fat-free ($p < 0.05$).

TABLE 6

Fatty Acid Composition of Cholesteryl Ester Fraction of Blood Plasma of Weanling Mice[a]

Diet	Palmitate (%)	Stearate (%)	Oleate (%)	Linoleate (%)	Saturated (%)
Chow	32.9(1.0)	2.9(0.6)	20.6(2.5)	36.8(5.9)	42.1(4.0)[d]
Fat-free	26.4(9.1)[d]	11.3(6.3)[e]	39.9(10.2)[c]	4.5(3.9)[d,f]	44.1(17.4)[d]
GMP	55.4(6.7)[b]	11.9(1.6)[b]	17.9(4.8)[b]	3.1(2.7)[b]	73.3(9.1)
GMP + SO	34.9(12.9)[d]	8.5(5.5)	13.5(3.5)	32.1(19.1)[d]	46.1(21.0)[d]
GMP + 18:1	33.2(6.1)[d]	5.8(1.8)[c,d]	36.9(3.6)[c,d]	4.1(2.9)[d,f]	42.[illegible][d]

[a]Values are means with standard deviations in parentheses. Number of mice and diets: GMP, 6 mice fed 30% palmitoyl glyc[illegible]MP + SO, 6 mice fed 30% palmitoyl glycerol + 4% safflower oil; GMP + 18:1, 4 mice fed 30% palmitoyl glycerol + 4% oleic a[illegible]w, 4 mice fed standard commercial pelleted diet; fat-free, 9 mice fed the GMP diet with palmitoyl glycerol replaced by sucrose. Blo[illegible]ma was extracted and the cholesteryl esters were isolated by thin layer chromatography. Fatty acid composition was determined by gas-liquid chromatography of the methyl esters. Only those fatty acids present in more than trace amounts are shown. The mean percent of myristate for the diets ranged from 2.7% to 6.4%, with no significant difference between them.

[b]Statistically different from chow ($p < 0.01$).

[c]Statistically different from chow ($p < 0.05$).

[d]Statistically different from GMP ($p < 0.01$).

[e]Statistically different from GMP ($p < 0.05$).

[f]Statistically different from fat-free ($p < 0.01$).

[g]Statistically different from fat-free ($p < 0.05$).

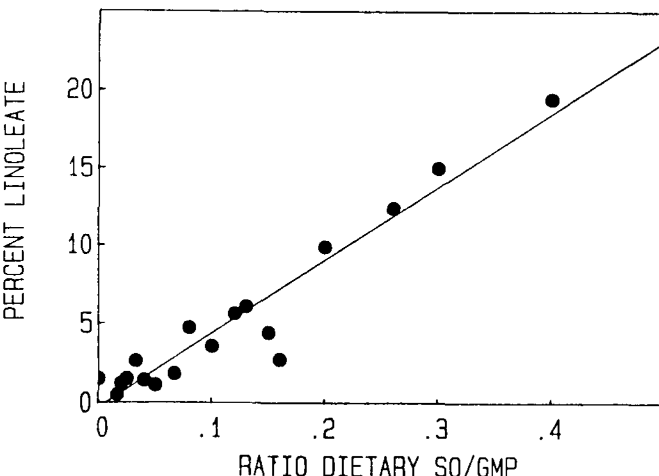

FIG. 1. Percent of linoleate in the triacylglycerol fraction of intestinal lymph from weanling mice fed various combinations of 0, 10, 15, 25 and 30% *rac*-1(3)-palmitoyl glycerol (GMP) plus 0, 0.5, 1, 2, 3 and 4% safflower oil (SO) for three days. Ratio dietary SO/GMP represents the ratio of dietary safflower oil to palmitoyl glycerol fed to the mice. Each point represents an average of one to five ratios of dietary safflower oil to palmitoyl glycerol. The value for each dietary combination was derived from an average of lymph from three to six mice.

When supplemental oleate was given, the percentage of oleate was elevated to the same level as that of linoleate when safflower oil was the supplement. However, when oleate was fed, the proportion of linoleate in the cholesteryl ester fraction was not higher than when only palmitoyl glycerol was fed. The net results of the changes in fatty acid composition were similar; namely that when mice were given a supplement of an unsaturated fat, the percentage of the total saturated fatty acids in this fraction was no greater than that in mice fed chow or mice fed a fat-free diet.

DISCUSSION

Most striking was the extremely high proportion of saturated fatty acids in the mouse lymph. In weanling mice, the proportion of total saturated fatty acids in each lipid class, other than the triacylglycerols, was more than 70%, irrespective of diet. When the mice were fed 30% *rac*-1(3)-palmitoyl glycerol, with or without 4% safflower oil, the sum of the saturated fatty acids was even higher, often approaching 90%. Although this might be expected for animals consuming saturated fat, the degree of saturation is higher for mice than that observed in other animals consuming high amounts of saturated fats. Chu and Hegsted (8) found a similar percentage of saturated fatty acids in the triacylglycerols of intestinal lymph of adult rats given palmitate, but the percentages of saturated fatty acids in the cholesteryl ester and phospholipid fractions were lower than we observed in mice fed either a palmitoyl glycerol diet or chow. Feldman et al. (9) reported lower percentages of saturated fatty acids in whole lymph of rats fed either palmitate or stearate. Even in ruminants, where the dietary fatty acids are largely saturated because of biohydrogenation occurring in the rumen, proportions of saturated fatty acids in the cholesteryl esters and phospholipids were not as great as we found in the corresponding fractions of mouse lymph.

Mouse lymph may not always be as highly saturated. In experiments where the proportions of dietary linoleate and palmitate were varied, the percentage of linoleate increased linearly with the ratio of linoleate to palmitate. However, the percentage of palmitate remained relatively constant. Hence it would appear that in mice, dietary unsaturated fatty acids influence lymph to a much greater degree than dietary saturated fatty acids.

Typically, the triacylglycerol fraction is expected to be the major lipid in lymph when animals ingest fat. This was observed with weanling and adult mice fed chow and with adult mice fed palmitoyl glycerol plus safflower oil. However, when weanling or adult mice were fed 30% palmitoyl glycerol, the cholesteryl ester fraction predominated. The free fatty acid fraction was greater than the triacylglycerols in these groups of mice, as well as in weanling animals fed palmitoyl glycerol and safflower oil. The diacylglycerol fraction was also increased when these diets were fed, although to a lesser degree. The findings would suggest that when mice were fed the palmitoyl glycerol, there was either a decrease in the synthesis of triacylglycerols or an increase in lipolytic activity.

In blood plasma, the cholesteryl ester fraction was greater than the triacylglycerol fraction when weanling mice were fed a low-fat diet, chow or a fat-free diet. This is consistent with studies that showed elevated triacylglycerols in rats fed a high-fat diet compared to chow (7). Although supplementing the palmitoyl glycerol diet with safflower oil affected the lipid composition of the lymph, subsequent metabolism eliminated these differences, and the proportions of the various plasma lipids were the same in weanling mice fed palmitoyl glycerol with or without the unsaturated fat.

As expected, the plasma lipids from weanling mice fed a palmitoyl glycerol diet were more saturated than from those fed chow or a fat-free diet. Unexpectedly, we found only traces of arachidonic acid in any lipid from the plasma of the weanling mice. It is noteworthy that, except for the cholesteryl ester fraction, there was very little difference in fatty acid composition of the plasma lipids between mice fed palmitoyl glycerol alone or with a protective unsaturated fat. On the other hand, the composition of the cholesteryl ester fraction paralleled the overall effects of the palmitoyl glycerol toxicity. The palmitate percentage and total proportion of saturated fatty acids were high only when the mice were fed the unsupplemented palmitoyl glycerol diet. When 4% safflower oil was the supplement, linoleate was increased but oleate was unchanged. Conversely, when 4% oleate was the supplement, oleate increased but linoleate was no higher than in the unsupplemented diet.

Although weanling mice were given a fat-free diet, the length of the experiment was too short for the classical syndrome of an essential fatty acid deficiency to appear. In these mice, the stearate was higher and the oleate lower in plasma triacylglycerols than was found in four-month-old rats given a fat-free diet (6). We also observed that the lymph lipids from weanling mice fed chow were higher in stearate and lower in oleate than those from adult mice fed chow. In studies with adipose tissue (11), the same trend was observed with the triacylglycerols from weanling versus adult mice. While it is possible that these differences resulted from an increased accumulation of oleate relative to stearate as the animals aged, it is also

possible that the fatty acid desaturases were not as active in the weanling mice. Prasad and Joshi (12) found that in chickens the desaturases developed with age. Additionally, the failure to find more than trace amounts of arachidonate in the plasma of weanling mice is consistent with this hypothesis.

The primary purpose for conducting this study was to lead to an explanation of the toxicity of dietary palmitoyl glycerol to mice. Acylglycerols of other saturated fatty acids, myristic acid (13) and stearic acid (14), were also toxic, as was *sn*-diacetyl-2-palmitoyl glycerol (2). Hence, the toxicity was not specific to *rac*-1(3)-palmitoyl glycerol but could be caused by ingestion of any saturated monoacylglycerol. At first sight, the toxicity may seem to be an acute form of an essential fatty acid deficiency. The toxic diet does not contain an essential fatty acid, and when linoleate is added to the diet the toxicity is ameliorated. Moreover, the addition of saturated fat to a diet deficient in essential fatty acids accentuates the syndrome of an essential fatty acid deficiency (15). However, there are several factors that negate this idea. Prefeeding linoleate for three days was ineffective in preventing the toxicity (2). Dietary oleate was as effective as linoleate in protection (14). Although it is conceivable that dietary oleate might release a supply of an essential fatty acid from some inactive pool, no change in linoleate or arachidonate was observed in plasma when oleate was fed.

With regard to the specific compound responsible for the toxicity, we had considered three possibilities (1): free palmitic acid from the palmitoyl glycerol, the monoacylglycerol itself and some metabolic product derived from palmitoyl glycerol (1). The first had been eliminated, since free palmitic acid was not as toxic as palmitoyl glycerol (1), and diacetyl, dipropionyl and dibutyryl derivaties of palmitoyl glycerol were much less toxic than *rac*-1(3)-palmitoyl glycerol because of hydrolysis of the palmitate (1). Although the results of the current study showed no major differences in fatty acid composition associated with diet (except for the cholesteryl esters), they are significant in that they indicate that the monoacylglycerol per se is not the toxic agent. Thus this finding, coupled with the fact that *rac*-1(3)-oleoyl glycerol was an effective form of an unsaturated fat (14), points to the probability that the immediate toxic agent is a metabolic product of palmitoyl glycerol.

Lipids of more than 70% saturated fatty acids would not be expected to be in a fluid state at the body temperature of a mouse. However, Bennett-Clark et al. (16) have shown that chylomicrons containing a high proportion of saturated fatty acids were produced as supercooled liquids. When they were held at temperatures below 23 C, they lost their spherical shape and appeared flattened and polygonal. There is an effect of ambient temperature on the toxicity of palmitoyl glycerol (3), so it is possible that altered chylomicron structure could be associated with the toxicity. On the other hand, there were no differences in the degree of saturation of lymph between mice that succumbed from consuming palmitoyl glycerol and those given 4% safflower oil that survived. Moreover, even though mice were ingesting diets containing 30% fat, cloudy mouse plasma was never observed, suggesting that the rate of chylomicron clearance is very high.

Except for the cholesteryl esters, there were basically no differences in the fatty acid composition between mice fed the toxic palmitoyl glycerol diet or the diet supplemented with a protective unsaturated fat. Furthermore, decreasing the toxic effects by reducing the percent of *rac*-1(3)-palmitoyl glycerol in the diet to 15% did not significantly affect the fatty acid composition of the plasma lipids (11). Therefore, it seems unlikely that the toxicity was related to the total percentage of saturated fatty acids in the plasma. However, the correlation of the fatty acid composition of the cholesteryl esters with the toxicity does suggest that this plasma lipid may be involved in the mechanism responsible for the toxicity of palmitoyl glycerol. Assuming that a similar correlation would be seen with the other toxic monoacylglycerols, it is possible that the cholesteryl ester fatty acid is a precursor for some vital membrane domain that loses its function when saturated. Extensive analysis of both fatty composition and incorporation of radioactivity from *rac*-1(3)-[1-^{14}C]palmitoyl glycerol (2) failed to show any differences between tissues of mice fed palmitoyl glycerol alone or with a protective unsaturated fat. However, it is possible that the defect may lie in microdomain(s) of a membrane that may not have been detectable in the gross composition studies conducted. Where such domain(s) might exist is unknown, but since death is so sudden following ingestion of the saturated monoacylglycerol, it seems likely that it lies in a vital organ.

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Effect of Cupric Ions on Serum and Liver Cholesterol Metabolism

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Cupric ions were administered subcutaneously to male Sprague-Dawley rats at a single dose of 200 μmol/kg. At 24 hr after administration, a remarkable increase of total and free cholesterol was seen in the rat serum. Also, when lecithin-cholesterol acyltransferase (LCAT) (E.C. 2.3.1.43) activity was expressed as the percentage of the total serum that free cholesterol esterified, the acyltransferase activity in rats treated with cupric ions showed a slight decrease while the triglyceride content in rat serum and liver decreased by 54% and 61%, respectively. However, the content of hepatic cholesterol in rats treated with cupric ions did not show such a marked change.

On the other hand, acid cholesteryl ester hydrolase activity (Acid CEH) (E.C. 3.1.1.14) in liver lysosomes of rats treated with cupric ions showed a marked decrease with increasing cupric ion concentration both in vivo and in vitro. Furthermore, cupric ions caused a marked release of the lysosomal enzymes cathepsin D and β-glucuronidase into the cytosolic fraction. The changes in acid cholesteryl ester hydrolase activity induced by cupric ions appear to be a direct effect of cupric ions on the enzyme. These results suggest that excessive cupric ion concentrations could cause various disorders in lipid metabolism.
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Cupric ions have been recognized as an essential trace element for hematopoiesis (1). Dietary cupric ion deficiency in rat has been shown to produce hypercholesterolemia (2–4). Moreover, several studies have indicated that diets high in zinc:curpic ion ratios are associated with hypercholesterolemia in rat (5,6). However, the role of cupric ions in cholesterol metabolism still is unknown.

The metabolism of cholesterol and cholesteryl esters in serum have been studied extensively. It also has been reported that the synthesis and turnover of bile acids correlate inversely with serum cholesterol in hypercholesterolemia (7,8). In contrast, it has been suggested that the marked accumulation of cholesteryl esters in hypercholesterolemia may be responsible for the structural change in lysosomal membranes that accompanies the decrease in acid cholesteryl ester hydrolase (Acid CEH) activity (9). Although several studies have characterized the activity and properties of lysosomal Acid CEH from liver (10–12), the association of lysosomal Acid CEH with the lipid of serum and liver has not been examined in any detail.

Therefore, we investigated the changes occurring in serum lipid levels, serum lecithin-cholesterol acyltransferase (LCAT) activity and lysosomal Acid CEH activity in cupric ion-treated rats.

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Abbreviations: Acid CEH, acid cholesterol ester hydrolase; BANA, α-N-benzoyl-DL-arginine-2-naphthylamide hydrochloride; EDTA, ethylenediaminetetraacetic acid; GLC, gas liquid chromatography; HDL, high density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDL, low density lipoprotein.

MATERIALS AND METHODS

Chemicals and radiochemicals. Cholesteryl [1-^{14}C]oleate (sp. act. 58.6 mCi/mmol) was purchased from New England Nuclear Corp. (Boston, MA). Phenolphthalein glucuronide, phenylphosphate, hemoglobin and α-N-benzoyl-DL-arginine-2-naphthylamide hydrochloride (BANA) were purchased from Sigma Chemical Co. (St. Louis, MO). Assay kits (free cholesterol C-Test Wako and HDL-cholesterol-Test Wako) were purchased from Wako Pure Chemical Ind. (Osaka, Japan).

Treatment of animals. Young male Sprague-Dawley rats weighing 150–180 g were used for all studies. Rats were given subcutaneously 0.1 ml of saline containing copper chloride (200 μmol/kg body) at a single dose (6 a.m.). At 24 hr after administration, the blood and liver were taken.

Preparation of subcellular fractions. For the preparation of lysosomal and cytosolic fractions from various tissues, the method of Brecher et al. (13) was followed. Rats were killed by guillotine, and the livers were perfused with ice-cold 1.15% KCl solution at 4 C. The liver was homogenized in eight volumes of ice-cold 0.25 M sucrose/1 mM ethylenediaminetetraacetic acid (EDTA)/0.01 M Tris-HCl buffer (pH 7.5). The homogenate was centrifuged at 1,000 × g for 10 min, and the resulting supernatant was centrifuged at 3,300 × g for 20 min. The 3,300 × g supernatant solution was centrifuged at 12,000 × g for 35 min. The pellet was rehomogenized in 0.25 M sucrose solution and recentrifuged at 12,000 × g for 20 min. The resulting pellet was resuspended in 0.25 M sucrose/0.01 M Tris-HCl buffer (pH 7.4). The original 12,000 × g supernatant solution was recentrifuged at 105,000 × g for 60 min to obtain the microsomal and cytosolic fractions.

Lecithin-cholesterol acyltransferase assay. LCAT activity was determined by the method of Nakagawa et al. (14). The standard assay medium contained 0.2 ml of rat serum and 0.3 ml of phosphate buffer (pH 7.4, ionic strength 0.1). The samples were placed in 15 ml screw-capped tubes, which then were flushed with N_2, sealed and incubated at 37 C for two hr with mechanical shaking. Free cholesterol in the reaction mixtures before and after incubation was determined with the Free Cholesterol C-Test Wako kit based on an enzymatic assay.

Acid cholesteryl ester hydrolase assay. The Acid CEH activity was measured by the method of Brecher et al. (13). Benzene solutions of cholesteryl oleate and cholesteryl [1-^{14}C]oleate were mixed, and the benzene was evaporated under N_2. Saline solution with 0.5% albumin was added, and the mixture was sonicated three 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 of 0.3 ml. 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) then was added. The solution was mixed for 25 sec by vortexing 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 aliquots to 10 ml of Aquasol 2 liquid scintillation mixture and counting the samples in an Aroka LSC 900 liquid scintillation counter.

Assay of lysosomal marker enzymes. Cathepsin D was assayed by the method of Hirado et al. (15), using hemoglobin as substrate. The amount of reaction products was assayed by the method of Lowry et al. (16). β-Glucuronidase was assayed with phenolphthalein glucuronide as substrate. Phenolphthalein liberated from the substrate was measured by the method of Gianetto and de Duve (17). Protein was assayed by the method of Lowry et al. (6).

Determination of cholesterol and triglyceride content. Total cholesterol and triglyceride in rat serum were determined by the method of Rudel and Morris (18) and van Handel and Zilversmit (19), respectively. Free cholesterol in rat serum was measured with the free cholesterol C-Test Wako Kit based on an enzymatic assay. High-density lipoprotein (HDL) cholesterol in rat serum was measured with the HDL cholesterol-Test Wako kit based on an enzymatic assay.

For determination of total and free cholesterol and triglyceride in rat liver, total lipids were extracted from rat liver homogenates by the procedure of Folch et al. (20). The chloroform extracts were evaporated to dryness, hydrolyzed with 5% methanolic-KOH at 60 C for one hr and extracted with hexane by the method of Uchiyama et al. (21). The hexane extracts were evaporated to dryness, and total cholesterol content as its trimethylsilyl ether derivative was determined by gas liquid chromatography (GLC) as described by Marcel and Vezina (22). Also, the chloroform extracts were evaporated to dryness, and the amount of free cholesterol and triglyceride were determined by the method mentioned above, respectively.

RESULTS

The effects of cupric ions on serum lipids content are shown in Table 1. At a single dose of 200 μmol/kg, the amount of total cholesterol in serum was increased markedly to about 150% of the control value. Also, the ratio of free to total cholesterol was increased in serum from rats receiving cupric ions. However, the serum triglyceride content showed a marked decrease to 55% of the control value. In contrast, the serum HDL cholesterol content showed no change following cupric ion treatment in comparison with the levels found in control rats.

It was thought possible that the increase of free cholesterol observed in cupric ion–treated rats may have been dependent upon a change in esterification activity. Therefore, the level of LCAT activity in serum was determined. If expressed as the percentage of the total serum cholesterol esterified, the LCAT activity in the serum of cupric ion–treated rats showed a slight decrease as compared to that of the control rats.

Changes in the cholesterol content of the liver also were investigated (Table 1). Total cholesterol content in rats receiving cupric ions was increased slightly to 120% of the control value. However, triglyceride content in the cupric ion–treated rats was reduced markedly to 60% of the control values. The time course of serum and liver cholesterol levels in cupric ion–treated rats also was studied. The levels of both reached a maximum after 12 to 24 hr and leveled off gradually until 48 hr (data not shown).

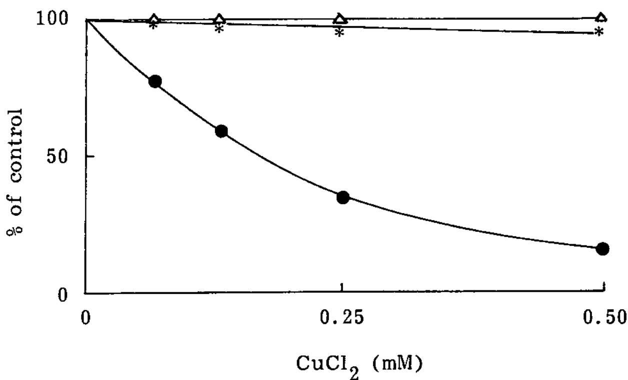

FIG. 1. Effect of cupric ions on lysosomal enzyme activities in vitro. The 100% values is the activity under standard assay conditions. ●, Acid CEH; △, cathepsin D; *, β-glucuronidase.

The effects of various cations on the cholesterol content of serum and liver are presented in Table 2. Serum cholesterol content in Cd^{2+}-treated rats was elevated slightly only at low concentration while Mn^{2+}, Co^{2+} and Zn^{2+} did not show any significant effect on serum and liver cholesterol contents.

Since it has been suggested that cupric ions are sequestered within the lysosome, the changes in cholesterol levels caused by cupric ion treatment may be mediated through this organelle. For this reason, the effects of cupric ions on liver lysosomal Acid CEH were studied. As shown in Table 3, when four different doses (400, 200, 40, 20 μmol/kg body wt) of cupric ions were administered to rats, cupric ions at doses more than 40 μmol/kg body wt caused an increase of cholesterol levels in serum and liver, and inhibition of Acid CEH activity at 24 hr after administration. This decrease occurred in a dose-dependent manner and ranged from 10 to 50% of control values.

The direct effect of cupric ions on lysosomal Acid CEH activity was investigated by incubation with lysosomes derived from normal rat liver (Fig. 1). Acid CEH was inhibited significantly at a cupric ion concentration of 0.25 mM in vitro, although the same concentration of cupric ions did not inhibit other lysosomal enzymes such as cathepsin D and β-glucuronidase. We studied the effect of cupric ions on Acid CEH activity in various tissues. Cupric ions had no effect on Acid CEH activity in the spleen, lung and heart (data not shown).

Lindquist (23) reported that cupric ions caused lysosome rupture and release of hydrolytic enzymes into the cytosolic fraction. Therefore, we studied the levels of lysosomal marker enzymes in the liver lysosomal and cytosolic fractions of both control and cupric ion–treated rats. The activities of the marker enzymes in lysosomes isolated from cupric ion-loaded livers showed a marked decrease of up to about 50% that of controls. On the other hand, the activities of cathepsin D and β-glucuronidase in the cytosolic fraction increased markedly (data not shown).

DISCUSSION

In our experiments, increases in cholesterol level and decreases in triglyceride level were observed in both the serum and liver of rats after administering cupric ions.

TABLE 1

Effect of Cupric Ions on Serum LCAT Activity and Serum and Liver Lipids

Serum	Control	$CuCl_2$
Total cholesterol (μg/ml)	726.6 ± 12.1	1071.4 ± 119.0[a]
Free cholesterol (μg/ml)	244.1 ± 32.8	427.0 ± 37.1[a]
Free/Total	0.33	0.41
HDL cholesterol (μg/ml)	456.3 ± 84.1	416.6 ± 28.0
Triglyceride (mg/dl)	49.65 ± 0.92	26.73 ± 0.46[a]
Esterification		
Net (μg/ml serum)	53.02 ± 1.04	80.14 ± 6.62[a]
% $\left(\frac{\text{cholesterol esterified}}{\text{free cholesterol}}\right)$	21.72 ± 1.52	18.77 ± 0.55[b]
Liver		
Total cholesterol (mg/g of liver)	1.396 ± 0.09	1.658 ± 0.04[b]
Free cholesterol (mg/g of liver)	0.900 ± 0.01	1.153 ± 0.01[b]
Free/Total	0.64	0.69
Triglyceride (mg/g of liver)	5.62 ± 0.90	3.43 ± 1.02[a]

Values are given as mean ± SD of six rats.
[a]Significantly different from control, $p < 0.01$.
[b]Significantly different from control, $p < 0.05$.

TABLE 2

Effect of Divalent Cations of Some Heavy Metals on Serum and Liver Cholesterol Contents

	Cholesterol	
Metals (μmol/kg)	Serum (μg/ml)	Liver (mg/g of liver)
None	70.51 ± 2.5 (100.0%)	1.463 ± 0.19 (100.0%)
$CuCl_2$ (200)	100.75 ± 5.0 (142.9%)[a]	1.734 ± 0.18 (118.4%)[b]
$CdCl_2$ (40)	87.75 ± 1.6 (124.4%)[a]	1.561 ± 0.38 (106.5%)
$MnCl_2$ (200)	74.25 ± 1.5 (105.3%)	1.238 ± 0.08 (84.5%)
$CoCl_2$ (200)	69.25 ± 1.2 (98.2%)	1.561 ± 0.23 (106.5%)
$ZnCl_2$ (200)	76.25 ± 1.7 (108.1%)	1.148 ± 0.07 (78.4%)

Values are given as mean ± SD of six rats. Values in parentheses are percentages of the values obtained in control rats (taken as 100%).
[a]Significantly different from control, $p < 0.01$.
[b]Significantly different from control, $p < 0.05$.

TABLE 3

Dose Response of Cupric Ions on Serum and Liver Lipid Contents and Acid CEH Activity

		$CuCl_2$ (μmol/kg)			
	Control	20	40	200	400
Serum cholesterol (mg/ml)	67.1 ± 8.6 (100.0%)	77.1 ± 0.1 (114.9%)	79.1 ± 2.8[a] (117.9%)	93.9 ± 5.2[a] (139.9%)	97.3 ± 6.0[a] (145.0%)
Liver cholesterol (mg/g of liver)	1.30 ± 0.1 (100.0%)	1.28 ± 0.1 (98.5%)	1.43 ± 0.1[b] (110.0%)	1.50 ± 0.1[b] (115.4%)	1.56 ± 0.1[b] (120.0%)
Liver triglyceride (mg/g of liver)	5.25 ± 0.1 (100.0%)	7.15 ± 1.5 (136.2%)	3.85 ± 0.2[a] (73.3%)	3.10 ± 0.2[a] (59.0%)	3.10 ± 0.5[a] (59.0%)
Acid CEH activity ($\times 10^5$ dpm/mg of protein/30 min)	5.62 ± 0.4 (100.0%)	5.06 ± 0.1[b] (90.0%)	3.65 ± 0.3[a] (64.9%)	2.81 ± 0.2[a] (50.0%)	2.81 ± 0.1[a] (50.0%)

Values are given as mean ± SD of six rats. Values in parentheses are percentages of the values obtained in control rats (taken as 100%).
[a]Significantly different from control, $p < 0.01$.
[b]Significantly different from control, $p < 0.05$.

The changes observed were concentration-dependent. Moreover, the ratio of free to total cholesterol in serum increased in cupric ion-treated rats in comparison to controls while HDL cholesterol was unchanged. These results suggest that the elevation of serum cholesterol content as a result of cupric ion treatment may be a cumulative phenomenon. Moreover, the high serum cholesterol in cupric ion-treated rats appears to be due to a selective elevation in serum low density lipoprotein (LDL).

On the other hand, Lei (2) and Allen and Klevay (3) have reported that cupric ion-deficient rats exhibited significant cholesterolemia, and plasma cholesterol showed a significant correlation with hepatic cupric ion concentration. Lefevre et al. (6) reported that cupric ion deficiency was associated with an increase in HDL cholesterol levels. Moreover, Harvey and Allen (24) indicated that the ratio of plasma cholesteryl esters to free cholesterol apparently was not reduced by cupric ion deficiency. These results demonstrate that dietary cupric ion deficiency or excessive levels of these ions can cause marked changes in serum cholesterol levels in rats. When LCAT was expressed on the basis of the percentage of cholesterol esterified, the levels of LCAT activity in the serum of rats treated with cupric ions were decreased slightly in comparison with those of control rats. In addition, according to the data in Table 1, free cholesterol content and the ratio of free to total cholesterol in serum of rats treated with cupric ions were increased compared to those of control rats. These results suggested that the increase in the free cholesterol content of serum from rats treated with cupric ions may have been due to the reduction of LCAT activity and the increase of secretion of total cholesterol from liver to serum. In contrast, Lau and Klevay (25) and Harvey and Allen (24) reported that cupric ion-deficient rats showed a significant decrease in LCAT activity, suggesting that cupric ions may be required for the synthesis or may be a constitutent of the enzyme. However, Morris and Church (26) have suggested that endogenous substrate concentration may be one of the most important factors in LCAT assay. As shown in Table 1, the content of free cholesterol in the serum was increased markedly following cupric ion treatment. These results suggest that elevation of net esterification in the serum of cupric ion-treated rats seems to have been due to an increase in the serum free cholesterol content.

On the other hand, Brown et al. (27), reporting on lipoprotein receptors in liver and on intracellular enzymes located in lysosomes found that the receptors play a special role in the regulation of serum cholesterol levels. Lindquist (23) reported an indirect effect of cupric ions on lysosomal enzymes. In our investigations with cupric ions, it is noteworthy that both lysosomes isolated from cupric ion-loaded liver and those isolated from normal rats and then treated with cupric ions in vitro showed a decrease in Acid CEH activity and an increase in the activity of free lysosomal enzymes in the cytosolic fraction.

These results suggested that the hypercholesterolemia induced by cupric ions may be related partly to a decrease of Acid CEH activity. Moreover, the inhibitory effects of cupric ions in vitro perhaps may occur through direct action on the Acid CEH molecule. However, the hypercholesterolemia induced by cupric ions may be related to proteolysis of the lipoprotein receptor due to the increase in the activity of free lysosomal protease in the cytosolic fraction. It is not clear now whether a reduction in Acid CEH by excessive cupric ion concentrations does contribute directly to hypercholesterolemia.

On the other hand, we have reported previously the presence of Acid CEH in body tissues and found high levels of activity in spleen, lung, liver and heart (12). The extent of the inhibitory effect of cupric ions on Acid CEH in the spleen, lung and heart was not nearly as marked as that in liver. These results suggest that the cupric ion-dependent inhibitory effect of Acid CEH may be a characteristic specific to the liver, which is the organ mainly concerned with the catabolism of cupric ions.

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Effects of Ketoconazole on Cholesterol Synthesis and Precursor Concentrations in the Rat Liver

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Ketoconazole, an antimycotic agent, given to rats for a week as 0.05% food addition had no effect on the hepatic concentrations of free and esterified cholesterol or on the activity of acyl coenzyme A: cholesterol-acyltransferase (ACAT). However, the levels of free methylated cholesterol precursors, especially lanosterols, less markedly $\Delta^{8,24}$ and Δ^8-dimethyl sterols and monomethyl sterols, were increased after only one day's treatment, while those of esterified methyl sterols were increased inconsistently, and those of free and esterified Δ^8-lathosterol, lathosterol and desmosterol were not affected at all. Cholestyramine treatment had no significant effect on ACAT in spite of a decrease in the hepatic content of esterified cholesterol and caused a marked increase in the free cholesterol precursor levels, especially in those of lathosterols. Cholestyramine given to ketoconazole-treated rats increased the hepatic levels of Δ^8 and Δ^7-lathosterols but not desmosterol or methylated cholesterol precursors. Ketoconazole increased and cholestyramine markedly decreased plant sterols, sitosterol and campesterol in the liver. In serum, the contents of both lanosterols and lathosterol were increased but that of cholesterol tended to be decreased by ketoconazole (−19%). The results indicate that ketoconazole impairs demethylation processes at C-14 and to some extent at C-4 in the rat liver, resulting in lowered serum cholesterol level.
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Ketoconazole, an imidazole derivative, is an effective oral antifungal drug, which currently is in use for the treating systemic mycoses (1). Its clinical effect is mediated mainly through an inhibition of C_{14}-demethylation of ergosterol synthesis (2). Furthermore, the accumulation of trimethylated sterols due to the inhibition of C_{14}-demethylation could disturb some membrane functions of non-mammalian cells (2,3)

At doses used for the treatment of mycoses, ketoconazole has been quite free from serious side effects, but it seems to affect cholesterol synthesis in mammalian cells. Studies in the rat have indicated that the hepatic C_{14}-demethylation is impaired (2). Clinical studies have revealed that ketoconazole inhibits steroid hormone synthesis (4,5) and also decreases serum cholesterol (6). The drop of serum cholesterol was associated with a marked elevation of serum lanosterol (7), suggesting that the inhibition of C-14 demethylation also could operate in human liver. However, the details of ketoconazole effects on hepatic cholesterol metabolism are unknown largely thus far. To clarify the effects of ketoconazole treatment on hepatic cholesterol synthesis, we have analyzed serum and hepatic concentrations of cholesterol and its sterol precursors and the activity of acyl coenzyme A:cholesterol acyltransferase (ACAT, EC 2.3.1.26) in rat liver under basal conditions and after cholesterol synthesis was stimulated by cholestyramine.

METHODS

Animals. Male Sprague-Dawley rats (Orion, Finland) were accustomed to a reversed-lighting cycle and killed in the middle of the 12 hr dark period. The animals were given ground rat chow (Hankkija, Finland) and tap water ad libitum. One gram of this chow contained 0.5 mg cholesterol and 0.4 mg sitosterol. In the treatment groups, chow was supplemented with 0.05% (w/w) ketoconazole for seven days (Janssen/Orion, Finland), 5% (w/w) cholestyramine resin (Lääkefarmos, Finland) for seven days or cholestyramine combined with ketoconazole for the final three days of the seven-day treatment. Previous experiments showed that cholestyramine treatment almost doubled fecal sterol output and quadrupled the activity of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase (EC.1.1.1.34), indicating activated cholesterol synthesis.

Analysis of lipids. After exsanguination of the rat under diethyl-ether anesthesia, serum was collected, the liver was removed, and a weighed portion was homogenized in chloroform methanol (2:1, v/v). Another portion was used for microsome preparation. After homogenization in a Dounce homogenizer, the sample was centrifuged for 15 min at 12,000 g, and the washed supernatant was further centrifuged 90 min at 105,000 g. The microsomal pellet was washed once, frozen in liquid N_2 and stored at −70 C for enzyme assays. The chloroform methanol extracts of hepatic and serum samples (8) were analyzed for free cholesterol, esterified cholesterol, its precursors, plant sterols, sitosterol and campesterol by thin layer chromatography (TLC)-gas liquid chromatography (GLC) methods using a SE-30 capillary column (9). The identification of GLC peaks was ascertained using mass spectrometric methods (10).

The SE-30 column does not separate dihydrolanosterol (DHL) from methostenol. The two precursors were separated in two samples by an additional TLC-run (Kieselgel 60 plastic plate in 55:45, v/v, diethylether heptane) before GLC. The precursors that were measured and their position in the cholesterol synthesis pathway are presented in Figure 1 (11). Systematic names of the precursors are presented in "Abbreviations."

Enzyme assays. The ACAT activity of the microsomal fraction was determined by following the incorporation of [1-^{14}C]oleic acid into cholesteryl ester (12). The

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Abbreviations: ACAT, acyl coenzyme A: cholesterol acyltransferase; campesterol (5α-cholest-24ξ-methyl-3β-ol); cholesterol (cholest-5-en-3β-ol), desmosterol (5α-cholest-5,24-dien-3β-ol); DHL, dihydrolanosterol (4,4,14α-trimethyl-5α-cholest-8-en-3β-ol); $\Delta^{8,24}$ DMS (4,4-dimethyl-5α-cholest-8(7), 24-dien-3-β-ol); Δ^8DMS (4,4-dimethyl-5-α-cholest-8-en-3β-ol); GLC, gas liquid chromatography; HMG-CoA, hydroxymethylglutaryl coenzyme A; lanosterol (4,4,14α-trimethyl-5α-cholest-8,24-dien-3β-ol); Δ^8lathosterol (5α-cholest-8-en-3β-ol); lathosterol (5α-cholest-7-en-3β-ol); Δ^8methostenol (4α-methyl-5α-cholest-8-en-3β-ol); methostenol (4α-methyl-5α-cholest-7-en-3β-ol); sitosterol (5α-cholest-24ξ-ethyl-3β-ol); $\Delta^{5,7}$-cholestenol (cholesta-5,7-dien-3β-ol); TLC, thin layer chromatography.

TABLE 1

Concentrations of Cholesterol and Phytosterols in Rat Liver during Ketoconazole and Cholestyramine Resin Treatment

	Cholesterol, mg/100 g wet weight			Phytosterols, μg/100 g wet weight					
				Campesterol			Sitosterol		
Group (n)	Free	Ester	%	Free	Ester	%	Free	Ester	%
Control (7)	179 ±7	19 ±2	10 ±1	3081 ±164	153 ±19	5 ±1	2836 ±142	188 ±13	6 ±1
Ketoconazole seven days (13)	192 ±5	18 ±1	9 ±1	3647 ±221	165 ±10	5 ±1	3431[a] ±197	209 ±21	6 ±1
Ketoconazole + cholestyramine (7)	180 ±6	9[a,b] ±1	5[a,b] ±1	3100 ±1200	90[b] ±16	5 ±1	2606 ±643	220 ±17	9[a,b] ±1
Cholestyramine (7)	176 ±7	9[a] ±2	5[a] ±1	1471[a] ±69	83[a] ±10	5 ±1	1386[a] ±89	286 ±133	15 ±5

Ketoconazole (0.05%) and cholestyramine (5%) were mixed with standard rat chow. Cholesterol and phytosterols were analyzed using gas liquid chromatography as described in Methods. Mean ± S.E.
[a] $p < 0.05$ or less vs control animals.
[b] $p < 0.05$ or less vs ketoconazole alone.

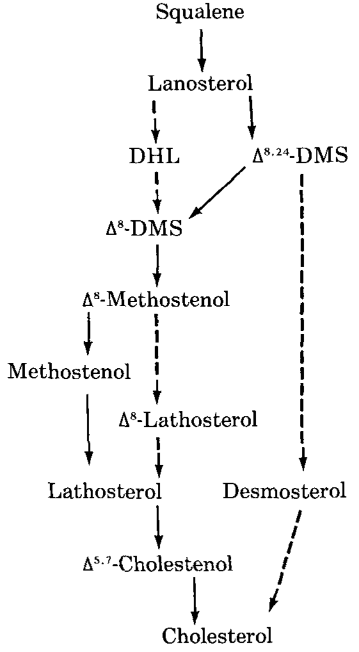

FIG. 1. Cholesterol synthesis from squalene. Solid arrows indicate major pathways, and broken arrows indicate probably weak pathways.

incubation volume was 0.2 ml, containing 100–200 μg microsomal protein and oleyl [1-^{14}C] coenzyme A (35 μM, SA 50 μCi/mol, New England Nuclear, Boston, MA). The incubation time was two min. The activity of the enzyme was linear with respect to time and protein concentrations under the experimental conditions. Microsomal protein was measured by the method of Lowry et al. (13).

Statistical methods. Control and treatment groups were compared using Student's t-test. P-values 0.05 or less were considered significant statistically.

TABLE 2

Activity of ACAT during Different Treatments

Group	Activity of ACAT, pmol/mg protein/min
Control	200 ± 15
Ketoconazole	178 ± 30
Ketoconazole + resin	158 ± 9[a]

Data are mean ± S.E. of three to four rats.
[a] $p < 0.05$ vs control.

RESULTS

Cholesterol content. Table 1 shows that treatment with 0.05% ketoconazole had no effect on the hepatic-free, esterified or total cholesterol contents. The percentage of esterification also was unaffected. However, cholestyramine treatment alone or in combination with ketoconazole reduced the concentration of hepatic esterified cholesterol significantly from 10% to 5%.

Plant sterol contents. In contrast to free and esterified cholesterol, hepatic contents of free plant sterols, campesterol and sitosterol tended to be increased by ketoconazole, clearly decreased by cholestyramine and virtually were unchanged by the combined cholestyramine-ketoconazole treatment (Table 1).

ACAT activity. Ketoconazole alone had no significant effect on hepatic ACAT activity, whereas in the cholestyramine-ketoconazole group the ACAT activity was lowered significantly as compared to the control group (Table 2).

Sterol precursors. The ketoconazole treatment for seven days increased the hepatic contents of all free methylated cholesterol precursors (Fig. 1). The contents of trimethylated lanosterol and DHL + methostenol were increased by a factor of 25 and eight, respectively, those of dimethylsterols increased from three-fold to six-fold, and

TABLE 3

Concentrations of Methylated and Demethylated Cholesterol Precursors in Rat Liver during Ketoconazole and Cholestyramine Resin Treatment

Lipid	μg/100 g wet weight			
	Control n = 7	Ketoconazole n = 13	Ketoconazole + resin n = 7	Resin n = 7
Lanosterol				
Free	43 ± 6	1086 ± 159[a]	1377 ± 367[a]	177 ± 36[a]
Ester	10 ± 2	44 ± 7[a]	26 ± 6	7 ± 1
Ester %	19 ± 3	5 ± 1[a]	2 ± 1[b]	4 ± 1[a,b]
DHL + methostenol				
Free	67 ± 19	463 ± 55[a]	1043 ± 389[a]	172 ± 32[a]
Ester	55 ± 11	108 ± 41	61 ± 15	47 ± 7
Ester %	49 ± 5	16 ± 5[a]	7 ± 1	23 ± 4[a]
DHL[c]				
Free	10 ± 3	320 ± 163	516 ± 128[a]	ND
Ester	10 ± 1	30 ± 2[a]	25 ± 1[a]	ND
Ester %	50 ± 9	11 ± 5[a]	5 ± 1[a]	ND
$\Delta^{8,24}$DMS				
Free	38 ± 7	108 ± 24[a]	128 ± 35	106 ± 20[a]
Ester	25 ± 4	23 ± 4	32 ± 5	26 ± 5
Ester %	39 ± 6	25 ± 4	26 ± 5	20 ± 1[a]
Δ^8 DMS				
Free	23 ± 5	138 ± 31[a]	116 ± 40	132 ± 53
Ester	7 ± 1	15 ± 4	10 ± 2	9 ± 2
Ester %	26 ± 5	10 ± 2[a]	14 ± 4	11 ± 3
Δ^8 methostenol				
Free	21 ± 5	62 ± 9[a]	92 ± 25[a]	97 ± 30[a]
Ester	19 ± 7	39 ± 6[a]	35 ± 12	17 ± 6
Ester %	45 ± 8	36 ± 3	22 ± 4[a]	17 ± 3
Methostenol[c]				
Free	62 ± 8	48 ± 21	79 ± 12	ND
Ester	47 ± 1	24 ± 4	25 ± 3	ND
Ester %	44 ± 3	36 ± 7	24 ± 5	ND
Δ^8 lathosterol				
Free	46 ± 8	49 ± 4	306 ± 102[a,b]	352 ± 28[a]
Ester	15 ± 3	17 ± 2	78 ± 7[a,b]	39 ± 6[a]
Ester %	25 ± 2	26 ± 2	13 ± 1	10 ± 2[a]
Lathosterol				
Free	320 ± 67	464 ± 35	2918 ± 922[a,b]	1662 ± 104[a]
Ester	56 ± 11	74 ± 9	83 ± 16	67 ± 12
Ester %	15 ± 2	14 ± 2	4 ± 1[a,b]	4 ± 1[a]
Desmosterol				
Free	189 ± 41	222 ± 18	251 ± 38	419 ± 48[a]
Ester	36 ± 8	32 ± 2	57 ± 8	27 ± 4
Ester %	16 ± 2	13 ± 1	15 ± 3	6 ± 1[a]

Data are mean ± S.E. Precursors were analyzed using thin layer chromatography before gas liquid chromatography; DHL, dihydrolanosterol; DMS, dimethylsterol; ND, not determined.

[a] $p < 0.05$ or less vs control.

[b] $p < 0.05$ or less vs ketoconazole alone.

[c] Analyzed separately in two samples, as described in Methods.

Δ^8-methostenol increased three-fold. The separation of DHL and methostenol in two samples suggested that ketoconazole increased DHL but not methostenol. Effects of ketoconazole on demethylated precurors, lathosterol, Δ^8-lathosterol and desmosterol were inconsistent. Virtually similar results were obtained after one day's treatment (data not shown). Ketoconazole affected esterified sterols less consistently but increased the levels of esterified lanosterols and Δ^8-methostenol. The slight response of the esterified methyl sterols to ketoconazole decreased the esterification percentage of methyl sterols while that of the demethylated precursors was unchanged (Table 3).

The treatment with resin alone increased markedly all the precursor concentrations, especially those of lathosterols. As compared to the ketoconazole and/or cholestyramine treatments, the simultaneous administration of the two agents had surprisingly little additional effect on precursor accumulation. However, the levels of desmosterol were decreased; the levels of lanosterols and lathosterol were increased more than expected from the combined effects of only the two drugs.

Serum sterols. The complete analysis of free and esterified precursors was performed only in one serum sample at each treatment group. The results not shown here suggested that the changes in methylated precursor levels

TABLE 4

Concentrations of Total Cholesterol and Its Precursors in Rat Serum during Ketoconazole and Cholestyramine Resin Treatment

	μg/dl (cholesterol mg/dl)			
Lipid	Control n = 7	Ketoconazole n = 9	Ketoconazole + resin n = 6	Resin n = 3
Lanosterol	23 ± 3	137 ± 22[a]	251 ± 57[a]	17 ± 2
DHL[c]	45 ± 7	129 ± 28[a]	92 ± 23	30 ± 2
Δ^8-lathosterol	32 ± 5	50 ± 5[a]	224 ± 33[a,b]	123 ± 6[a]
Lathosterol	74 ± 5	152 ± 9[a]	317 ± 8[a,b]	121 ± 8[a]
Desmosterol	177 ± 20	282 ± 16	334 ± 9	144 ± 27
Cholesterol	74 ± 8	60 ± 4	41 ± 2[a,b]	41 ± 4[a]

Data are mean ± S.E. Lipids were analyzed using gas liquid chromatography as described in Methods without separation of free and esterified fractions.

[a]$p < 0.05$ or less vs control.

[b]$p < 0.05$ or less vs ketoconazole alone.

[c]Includes methostenol.

(free and esterified) were similar to those observed in the liver with conspicuous elevations in the contents of lanosterols. Total levels of precursors and cholesterol were measured in a larger group of animals (Table 4), and the results showed that in addition to lanosterol and DHL the lathosterols were elevated in rat serum during ketoconazole treatment. Total cholesterol, in turn, tended to be decreased by ketoconazole; the decline was 45% during the combination treatment. However, cholestyramine alone also seemed to decrease cholesterol, whereas lathosterols were increased significantly as compared with control rats. Lanosterols were increased only in terms of μg/mg of serum cholesterol.

DISCUSSION

Accumulation of hepatic cholesterol precursors by ketoconazole treatment in this study suggests that the drug principally affects two steps of cholesterol synthesis in the rat liver. As in yeast cells, the demethylation of C-14 seems to be the major point of inhibition. In addition, also 4α-demethylation and possibly the conversion of Δ^8 to Δ^7 are affected because Δ^8-methostenol accumulates more than methostenol. Furthermore, it should be noted that the contents of lathosterols, which were not affected by ketoconazole in the liver, were elevated by the drug in serum, suggesting that the conversion of Δ^8-lathosterol to Δ^7-lathosterol and further to $\Delta^{5,7}$-cholestenol might also be altered by ketoconazole. In man, large doses of ketoconazole resulted in an accumulation of free lanosterols and to a lesser extent of Δ^8 precursor sterols in serum and bile, suggesting that in addition to 14α-demethylation the conversion of Δ^8-double bond to Δ^7-double bond was inhibited (14). The reduction of Δ^{24}, which is inhibited by triparanol (11), probably is not inhibited by ketoconazole because the desmosterol content was unchanged. However, the desmosterol pathway (see Fig. 1) with the preserved side chain double bond may be inhibited to some extent as a whole because accumulation of lanosterol results in its enhanced conversion to dihydrolanosterol. Thus, predominance of the side chain-saturated pathway of lanosterol metabolism may explain the unchanged desmosterol content in serum and liver during the ketoconazole-resin treatment.

Of the reactions involved in the conversion of lanosterol to cholesterol, 14α-demethylation is cytochrome P450-linked, 4α-demethylation is catalyzed by a mixed function oxidase (methyl sterol oxidase) without cytochrome P450 requirements (11,15), and the synthesis of methostenol from Δ^8 methostenol is catalyzed by microsomal Δ^8 isomerase (16). Thus, the results extend the earlier conception of ketoconazole as a general inhibitor of cytochrome P450-dependent enzymes (17).

It is of interest that despite the clear accumulation of the cholesterol precursors and the decrease of the serum cholesterol concentration, hepatic cholesterol concentrations were not decreased. This can be explained by the short treatment period (seven days), the presence of cholesterol in the rat diet, reduced biliary secretion or possibly by a concomitant inhibition of cytochrome P450-linked cholesterol 7α-hydroxylase or some other hydroxylase of bile acid synthesis in the rat liver. In view that plant sterols reaching the liver are partly eliminated as bile acids (18), their rise by ketoconazole and the decrease by cholestyramine suggest that the bile acid synthesis also could be inhibited by ketoconazole. In fact, human studies have indicated that the drug inhibits chenodeoxycholic acid synthesis (14) while in the rat the drug appears to inhibit cholic acid and β-muricholic acid synthesis also (19).

Depletion of hepatic cholesterol by cholestyramine enhances cholesterol synthesis when the activity of HMG-CoA reductase and methyl sterol oxidase are stimulated (20). This results in an increase in the hepatic and serum squalene and methylsterol contents (21) and, as shown in this study, especially in demethylated precursor concentrations. Accordingly, it was expected that inhibition of this flow by ketoconazole at lanosterol and at later possible steps might deplete dramatically hepatic cholesterol and result in a marked accumulation of methylated cholesterol precursors in the liver. However, the combination of cholestyramine and ketoconazole did not increase conspicuously the hepatic or serum contents of methyl sterols above the expected additional levels. Thus, the

stimulation of cholesterol synthesis by cholestyramine may partly overcome the synthesis blockade induced by ketoconazole. Further accumulation of Δ^7-lathosterol and Δ^8-lathosterol by the combination therapy was quite similar to that during cholestyramine alone and suggests that ketoconazole does not affect double bond formation at C-5.

The activity of ACAT in this study deserves some comment. In the liver, the activity of ACAT was unchanged by ketoconazole treatment, and the esterified precursor sterols generally did not increase proportionately to free sterols. This led to a decreased percentage of esterification of lanosterols especially but also Δ^8DMS. This can be due to low esterification of $4\alpha 4\beta$ methylated intermediates because these sterols are poor substrates for hepatic ACAT (22).

This study demonstrates the complexity of effects of ketoconazole on sterol biosynthesis in mammalian liver and suggests that ketoconazole could offer a tool to study the regulation of the synthesis beyond lanosterol. Furthermore, the hypocholesterolemic action of ketoconazole, observed especially in man (7,14), may imply a role for imidazole derivatives in the treatment of hyperlipidemias, possibly in combination with bile acid-binding resins.

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Polyunsaturated Fatty Acids in Tissues of Rats Fed Trielaidin and High or Low Levels of Linolenic Acid

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Male and female weanling rats that were born to dams fed a diet low in linolenic acid received diets of 15% lipids by weight containing 45% elaidic acid (as trielaidin) and 8.5% or 0.1% linolenic acid for 10 weeks. Four other groups, in which palmitic or oleic acid replaced elaidic acid in the diet, served as controls. The fatty acid profiles of several lipid classes were determined in adipose tissue, adrenals, testes, heart and brain. Elaidic acid was incorporated into tissue lipids in varying degrees, depending on the organ and on the lipid class. Feeding elaidic acid induced no changes in the polyunsaturated fatty acid (PUFA) profiles of testes lipids but resulted in definite modifications of the PUFA patterns of heart phosphatidylcholine (PC) and phosphatidylethanolamine (PE). In linolenic acid-deprived rats, arachidonic acid was decreased in PC and linoleic acid was increased in both PC and PE; 22:5n-6 was strongly depressed in both PC and PE. In linolenic acid-fed rats, 22:6n-3 was decreased in PC and PE. These changes, on the whole, were more evident in females, and some also were observed in adrenal cholesteryl esters but only slightly in brain phospholipids. The apparent inhibition of the biosynthesis of PUFA induced by dietary elaidic acid appeared to be complex and of greater intensity in the n-6 fatty acid series than in their n-3 homologues.
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During the process of hydrogenation used to produce hardened fat, the unsaturated fatty acids are isomerized, yielding a mixture of geometric (*cis* and *trans*) and positional isomers (1–3). The effects of isomeric fatty acids on the essential fatty acid (EFA) status of experimental animals and on the metabolism of EFA were the focus of nutritional research (4–12). In these works, linoleic acid and/or its metabolites were extensively studied. On the contrary, linolenic acid, a polyunsaturated fatty acid (PUFA) of high biological importance that possibly is essential for mammals (13) has not received as much attention. However, its dietary supply was controlled in many experimental works, especially the last ones (11,12). In a previous study (14), we investigated the effects of brassidic acid (n-9, *trans* docosenoic acid) and of elaidic acid (n-9, *trans* octadecenoic acid) on the fatty acid composition of organ lipids in rats fed a high or a low supply of linoleic acid. Dietary elaidic acid was found to induce changes in n-6 PUFA, especially in the heart. In this work, the incorporation of elaidic acid in body lipids and the effects of dietary elaidic acid on the PUFA profiles of several organs were studied in male and female rats fed diets with high or low linolenic acid contents.

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Abbreviations: ANOVA, analysis of variance; CE, cholesteryl esters; CL, cardiolipin; EFA, essential fatty acid; GLC, gas-liquid chromatography; PC, phosphatidycholine; PE, phosphatidylethanolamine; PHSO, partially hydrogenated soybean oil; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; TG, triacylglycerols; TLC, thin-layer chromatography.

MATERIAL AND METHODS

Animals and diets. Weanling Wistar female rats were fed a purified diet containing 15% sunflower oil by weight and 2% liver meal as sole lipid sources. Apart from the addition of liver meal, the composition of the diet was the same as previously published (15). This liver meal-supplemented diet contained about 100 g/kg of linoleic acid and 400 mg/kg of n-3 PUFA. After three months, the rats were mated, and the diet was continued during gestation and lactation. Twenty-four rats (12 males and 12 females) born to these dams were kept on the maternal diet three to six days after weaning. They then were divided randomly into six groups of four (two males and two females in each) and fed a purified diet ad libitum for 10 weeks. This diet differed from the maternal diet by the absence of liver meal and by 0.4% instead of 0.9% calcium. The digestibility of elaidic acid was 97–98% (15). The experimental diets differed from each other only in the fatty acid composition of the lipids (15% by weight). These were blends of trielaidin (containing 98% n-9, *trans*-octadecenoic acid), triolein, palm oil, sunflower oil and linseed oil in adequate amounts. The fatty acid composition of these mixtures is shown in Table 1. Linolenic acid was present in high amounts in three mixtures (PL, EL and OL) and in very low amounts (0.11–0.13% of dietary fatty acids, i.e. 160–200 mg/kg diet) in the three other mixtures (P, E and O). The sum of linoleic + linolenic acid contents was the same in all mixtures (ca. 21%). This experiment can be considered as a 2^3 factorial design; the factors are the sex of the rats, the dietary essential fatty acids (with or without linolenic acid) and the dietary nonessential fatty acids (elaidic, palmitic or oleic acid). At the beginning of the experimental period, control weanling rats were killed and their organs excised. Weight gain and food intake were recorded individually throughout the experiment. At the end of the 10-week period, the rats were killed; the heart, brain, testes, adrenals and a piece of perirenal adipose tissue were excised,

TABLE 1

Fatty Acid Composition (%) of Dietary Lipids

	Experimental groups					
Fatty acid	P	PL	E	EL	O	OL
16:0	36.7	37.0	8.8	9.0	8.4	8.8
18:0	4.5	4.5	2.3	2.2	2.2	2.0
18:1n-9 *trans*	—	—	43.8	45.1	—	—
18:1n-9 *cis*	34.3	34.9	21.9	21.7	65.9	66.3
18:2n-6	21.1	12.6	20.7	12.4	21.6	12.7
18:3n-3	0.11	8.3	0.13	7.9	0.10	8.4

weighed and stored at −20 C in chloroform/methanol (2:1, v/v) with 0.01% hydroquinone until analysis. In each group, the adrenals were pooled for every two rats of the same sex.

Analytical methods. Organs lipids were extracted according to Folch et al. (16), except adipose tissue, for which only chloroform was used. Neutral lipids and phospholipids of hearts, testes and adrenals were separated using Sep-Pak silica cartridges (17). These fractions were separated further in classes by thin-layer chromatography (TLC) on Silica Gel G, using hexane/diethyl ether/acetic acid (70:30:1, v/v/v) as migrating solvent for neutral lipids, and chloroform/methanol/14% ammonia in water (65:25:4, v/v/v) for phospholipids. Total brain lipids were separated directly by TLC (18). After spraying the plates with 0.02% Rhodamine B and visualizing the separated bands under UV light, adrenal triacylglycerols (TG), cholesteryl esters (CE), phosphatidylcholine (PC) and phosphatidylethanolamine (PE), testes TG, PC and PE, heart TG, PC, PE and cardiolipin (CL), and brain PC, PE and phosphatidylserine (PS) were scraped into teflon-lined, screw-capped tubes for methylation. Methyl esters were prepared by reacting the lipid classes at room temperature, in the presence of the silica gel, with 2 N sodium methoxide in methanol/benzene (60:40 v/v) for 10 min (except for CE: 30 min), then with an excess of 5% sulfuric acid in methanol for 30 min (adaptation of the method of Glass [19]). Water was added to the reaction mixtures, and methyl esters were extracted once with hexane and washed with water. Fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) using both Carbowax 20M- and Silar 10C-coated glass capillary columns (30m × 0.3 mm I.D.; isothermal analyses with the temperature ranging from 175 to 190 C). The first stationary phase gave a complete profile of all organ fatty acids with the exception of the geometrical isomers that were separated on Silar 10C columns. The gas chromatographs were equipped with Ross injection devices and FID detectors coupled to integrators.

Statistical analysis. Except for adrenal fatty acids, for which no statistical treatment was done, the data were treated by two- or three-way analysis of variance (ANOVA), with complete models (all interactions) (20). In many cases, the heterogeneity of variances between groups required two separate one- or two-way ANOVAs, on the groups with (PL, EL, OL) and without (P, E, O) linolenic acid. Additional methods (method of contrasts, of Newman and Keuls [20]), were used when needed to compare means (i.e. to compare the levels of the three-level factor, or to analyze interactions). All effects or differences described in Results are significant at the 5% probability level. Fatty acid percentages showed, as expected, relatively small within-group variations, allowing many effects or differences to be detected with only two rats per group. Calculations were made with a Commodore CBM 4032 microcomputer.

RESULTS

PUFA content of tissues at start of experimental period. As a consequence of the maternal diet, organ lipids of the weanling rats contained high amounts of n-6 PUFA and comparatively low amounts of n-3 PUFA (Table 2), except for brain PE and PS in which 22:6n-3 amounted to ca. 10%. Feeding dams a commercial stock diet, which generally contains substantial amounts of linolenic acid, would have resulted in high levels of n-3 PUFA in the organ lipids of the progeny (21). These n-3 PUFA, especially 22:6, are known for their relative persistence in body lipids, mostly in the brain (13), even if the post-weaning diet is low in linolenic acid. On the contrary, the tissue PUFA contents of (n-3)-depleted rats were much more sensitive to the subsequent feeding of linolenic acid. For this experiment, it seemed advantageous to use (n-3)-depleted weanling rats.

Incorporation of elaidic acid into organ lipids. Elaidic acid was present in high amounts in the TG of adrenals, testes and the heart, and even higher in adipose tissue

TABLE 2

PUFA of Heart Phospholipids of 24–29 Day-old Rats at the Start of the Experimental Period[a]

Fatty acid	Rats born to sunflower oil-fed dams[b]				Rats born to chow-fed dams[c]
	PC		PE		
	M	F	M	F	Total PL,M
20:4n-6	29.5	24.4	26.1	13.1	17.1
22:4n-6	5.2	3.4	3.2	4.4	0.9
22:5n-6	6.7	3.8	2.9	7.0	0.4
22:5n-3	1.1	0.8	0.5	0.5	3.2
22:6n-3	1.7	1.0	0.7	2.1	15.0

[a]Percentage of the fatty acids of each class; pools of 3–4 rats.
[b]This experiment.
[c]Previous work, see ref. 20.

TABLE 3

Incorporation of Elaidic Acid in Organ Lipids[a]

Organ	Lipid class	Experimental groups				Pooled SEM
		E		EL		
		M	F	M	F	
Adipose tissue	Total lipids	37.8	34.6	36.7	34.7	0.37
Heart	TG	30.6	35.6	30.7	32.3	1.50
	PC	23.5	25.6	23.2	22.1	0.98
	PE	30.0	32.4	31.5	27.3	0.94
	CL	2.3	1.7	1.9	3.5	0.43
Brain	PC	1.7	1.9	1.7	1.6	0.05
	PE	2.0	1.9	1.4	1.8	0.18
	PS	2.1	2.2	2.6	1.7	0.37
Testes	TG	28.5	—	28.9	—	1.89
	PC	1.2	—	1.1	—	0.07
	PE	1.0	—	1.1	—	0.12
Adrenals	TG	28.6	31.1	29.7	31.2	—
	CE	16.9	19.4	18.3	20.3	—
	PC	14.9	15.8	17.0	15.1	—
	PE	7.8	9.4	9.6	9.1	—

[a]Percentage of elaidate in each class; means of two rats, except in adrenals: pools of two rats.

(Table 3). In the latter, male rats incorporated slightly more elaidic acid than females. In all organ TG, the *trans* acid was incorporated at the expense of both saturated (mainly palmitic) and oleic acid. In addition to 9t-18:1, adrenal CE of elaidic acid-fed rats (E and EL groups) contained a fatty acid having the retention characteristics of *trans* 20:1 on both Carbowax 20M and Silar 10C columns (ca. 5-6%). The incorporation of elaidic acid into PC and PE was highest in the heart and significant in adrenals; it substituted almost exclusively for stearic acid. Comparatively, testes PC and PE contained very low amounts of the *trans* acid, as did heart cardiolipin and all phospholipid classes of brain. No effect of dietary linolenic acid or sex was detected in any organ or lipid class considered, except for the small difference between sexes observed in adipose tissue.

Fatty acid composition of adipose tissue. The fatty acid composition of adipose tissue reflected that of the dietary lipids, especially the elaidic, linoleic and linolenic acid contents. Minor changes also were observed: linoleic and linolenic acids were slightly higher in males than in females (linoleic acid: group P, E, O, 21.4% vs 18.5%, respectively; groups PL, EL, OL, 13.1% vs 11.9%, respectively; linolenic acid: groups PL, EL, OL, 5.1% vs 4.4%, respectively). Linoleic and linolenic acid were lower in oleic acid-fed rats than in rats fed palmitic or elaidic acid (linoleic acid: groups P, E: 20.6% vs group O: 18.5%; groups PL, EL: 13.1% vs group OL: 11.2%; linolenic acid: groups PL, EL: 5.0% vs group OL: 4.2%).

PUFA of adrenal lipids. The presence of linolenic acid in the diet decreased 22:4n-6 and 22:5n-6 and increased n-3 PUFA (20:5, 22:5, 22:6) in adrenal cholesteryl esters (Table 4). There were always more n-6 22 PUFA, but not n-3 PUFA, in females than in males. Among the linolenic acid-deprived rats, those fed elaidic acid had the lowest contents of 20:4n-6, 22:4n-6 and 22:5n-6. In the linolenic acid-fed series, n-3 PUFA (20:5, 22:5, 22:6) were higher in palmitic acid-fed rats (PL) than in elaidic- or oleic acid-fed rats (EL, OL). PUFA-levels in adrenal PC and PE were influenced by dietary linolenic acid and may be different in each sex, but showed no great changes due to elaidic acid.

PUFA of testis lipids. Rat testis lipids, including TG, were characterized by high contents of long-chain PUFA of the n-6 series, especially 22:5, but also 20:3, 20:4, 22:4, 24:4 and 24:5 (Table 5). Linoleic acid was depressed in TG by dietary linolenic acid (as in the TG of all other organs) but was slightly increased in PC and PE, as well as 20:3n-6. Dietary 18:3n-3 depressed 20:4n-6 in PC, and 22:4n-6 in PC and PE. However, 22:5n-6 and n-6 C24 PUFA remained unchanged in the three classes. Except for linolenic acid in TG, n-3 PUFA levels were low, even in linolenic acid-fed rats. Moreover, the PUFA contents of testis lipids were insensitive to the feeding of elaidic acid.

PUFA of heart lipids. In heart CL, a phospholipid class containing mainly linoleic acid, the oleic acid-fed rats had the lowest linoleic acid content: 64% (O) and 51% (OL) by comparison to 71% (P), 80% (PL), 86% (E) and 81% (EL). This decrease of linoleic acid was compensated by an increase of saturated and oleic acids, as well as of other

TABLE 4

PUFA of Adrenal CE[a]

Fatty acid	Sex	Experimental groups					
		P	PL	E	EL	O	OL
18:2n-6	M	3.6	3.6	4.6	3.8	3.3	3.2
	F	3.6	3.2	5.0	3.6	3.9	3.3
20:3n-6	M	3.0	3.2	3.1	2.8	3.4	2.3
	F	2.7	2.5	2.6	1.9	3.4	2.0
20:4n-6	M	16.7	6.7	6.6	6.9	12.9	7.9
	F	15.5	7.7	8.2	8.0	8.4	9.7
22:4n-6	M	19.9	5.7	17.3	7.4	20.5	9.0
	F	22.7	12.7	21.0	12.2	25.5	16.5
22:5n-6	M	6.7	0.8	2.7	1.1	4.8	1.3
	F	9.1	1.1	4.7	1.2	8.0	1.2
20:5n-3	M	—	3.6	—	2.4	—	2.2
	F	—	2.1	—	1.7	—	1.5
22:5n-3	M	0.8	13.8	0.3	8.3	0.7	8.9
	F	1.0	10.4	0.2	6.0	0.2	5.8
22:6n-3	M	1.2	10.1	0.4	3.8	0.4	6.4
	F	0.7	9.2	0.5	4.8	0.6	6.5

[a]Percentage of the fatty acids of the class; pools of two rats. Other PUFA not shown (<1.5%): 20:3n-9, 22:3n-9, 20:2n-6, 22:2n-6, 24:4n-6, 24:5n-6.

TABLE 5

PUFA of Testis Lipids[a]

Lipid class	Fatty acid	Experimental groups						Pooled SEM
		P	PL	E	EL	O	OL	
TG	18:2n-6	16.4	10.4	16.2	11.1	13.2	9.4	0.62
	20:3n-6	0.5	0.7	0.7	0.7	0.7	0.9	0.10
	20:4n-6	2.2	1.9	2.3	1.8	3.0	2.8	0.18
	22:4n-6	1.6	1.5	2.0	1.7	2.6	1.8	0.22
	22:5n-6	6.4	7.5	8.3	7.5	10.4	9.2	1.04
	24:4n-6	1.1	1.2	1.7	1.6	1.7	2.1	0.25
	24:5n-6	1.5	1.7	2.1	1.8	2.3	1.7	0.21
	18:3n-3	0.1	4.1	0.1	4.3	0.1	3.4	0.77
	22:6n-3	tr.[b]	0.5	tr.	0.2	tr.	0.4	0.03
PC	18:2n-6	5.4	7.1	6.7	8.0	4.5	6.7	0.32
	20:3n-6	1.8	2.2	1.7	2.3	1.4	1.9	0.21
	20:4n-6	18.5	14.2	17.7	15.1	17.5	15.1	0.55
	22:4n-6	1.4	1.0	1.3	1.2	1.3	1.1	0.08
	22:5n-6	16.8	14.2	14.9	14.6	15.1	14.5	0.77
	24:4n-6	1.3	1.4	1.4	1.6	1.4	1.4	0.12
	24:5n-6	0.9	0.9	0.8	0.9	0.9	0.8	0.04
	22:6n-3	0.1	0.7	0.1	0.7	0.1	0.7	0.02
PE	18:2n-6	5.0	7.0	3.6	4.9	2.8	4.1	0.28
	20:3n-6	1.0	1.3	0.6	0.9	0.6	0.8	0.06
	20:4n-6	24.6	22.5	23.2	23.2	25.8	23.2	0.95
	22:4n-6	4.0	2.9	3.3	3.1	4.0	3.1	0.25
	22:5n-6	31.6	26.6	25.7	26.4	30.1	26.8	1.66
	24:4n-6	1.3	1.0	1.0	1.0	1.2	1.0	0.09
	24:5n-6	1.1	0.9	0.9	0.9	1.3	0.9	0.10
	22:6n-3	0.3	1.7	0.3	1.6	0.4	1.6	0.04

[a]Percentage of the fatty acids of each class; means of two rats. Other PUFA not shown (<1%): 20:2n-9, 20:3n-9 and n-7, 18:3n-6, 20:2n-6, 22:3n-6, 20:5n-3, 22:5n-3.
[b]<0.1%.

PUFA. Elaidic- and palmitic acid-fed rats had similar fatty acid patterns in heart CL.

The 22:6n-3 contents of heart PC and PE were higher in females than in males for all dietary groups, as well as that of 22:5n-6 for groups P, E and O (Table 6). The inverse was true for 18:2n-6, which almost always was higher in males than in females. Feeding linolenic acid shifted both PC and PE fatty acid profiles towards n-3 PUFA (22:5 and 22:6), which were increased at the expense of n-6 PUFA (20:4, 22:4, 22:5). Contrary to other organs, the PUFA profiles of heart phospholipids strikingly responded to elaidic acid feeding: linoleic acid was raised in both PC and PE of groups E and EL; 22:6n-3 was lowered in both PC and PE of linolenic acid-fed rats. Those fed palmitic acid had the highest content 22:6n-3 in PE (PL > OL > EL). In linolenic acid-deprived rats, dietary elaidic acid depressed 22:4n-6 and 22:5n-6 in both PC and PE, as well as arachidonic acid in PC. The latter PUFA was increased in the PE of linolenic acid-deficient, elaidic acid-fed females (E vs P and O). The most patent of these effects is the drop of PE 22:5n-6 content in the elaidic acid-fed, linolenic acid-deprived rats (group E).

The inhibition of n-6 PUFA synthesis also was seen in heart TG of linolenic acid-deprived rats: arachidonic acid contents were lower in group E (males: 2.4%; females: 1.6%) than in the other groups (group P: 4.5% and 4.0%; group O: 4.0% and 2.8%, respectively); 22:5n-6 was particularly high in group P (7.7% and 9.2%, respectively), present in group O (0.4% and 1.1%, respectively) and nearly absent in group E (0.3% and 0.0%, respectively).

PUFA of brain phospholipids. Differences between males and females in the fatty acid profiles of brain PC, PE and PS either were absent or very slight. For this reason, the data presented are the means of the dietary groups (two males and two females). Feeding of linolenic acid resulted in the replacement of the n-6 PUFA by 22:6n-3 in brain phosphoglycerides. The three n-6 PUFA were not affected equally: arachidonic acid and 22:4n-6 were only slightly reduced by dietary 18:3n-3 but 22:5n-6 underwent a sharp decrease. For a given class, the sum of all PUFA percentages remained remarkably constant, despite significant but small effects of dietary factors in PC and PE. The presence of elaidic acid in the diet had little influence on brain lipid PUFA. However, the slight but significant decrease of 22:5n-6 in PE and PS of linolenic acid-deprived, elaidic acid-fed rats (group E) is noteworthy.

TABLE 6

PUFA of Heart Phospholipids[a]

Lipid class	Fatty acid	Sex	Experimental groups: P	PL	E	EL	O	OL	Pooled SEM
PC	18:2n-6	M	11.0	14.3	12.4	14.9	8.2	12.8	0.83
		F	6.2	10.7	12.4	13.0	5.6	7.8	
	20:4n-6	M	34.6	18.7	28.4	17.4	32.5	20.9	1.06
		F	35.4	22.1	26.9	19.3	33.3	22.7	
	22:4n-6	M	1.3	tr.[b]	0.9	0.1	1.4	tr.	0.05
		F	1.6	0.1	0.8	0.2	1.4	0.1	
	22:5n-6	M	1.3	—	0.3	0.1	1.3	tr.	0.11
		F	3.1	—	0.7	tr.	3.6	tr.	
	22:5n-3	M	0.2	3.8	0.2	3.5	0.1	3.2	0.68
		F	0.2	4.2	0.2	2.6	tr.	2.8	
	22:6n-3	M	0.4	4.6	0.4	2.6	0.6	3.9	1.05
		F	0.6	5.4	0.7	5.0	0.9	7.2	
PE	18:2n-6	M	6.3	5.8	7.1	6.5	5.5	5.3	0.41
		F	3.5	4.5	6.6	5.3	3.6	3.3	
	20:4n-6	M	27.8	16.3	29.3	19.4	23.3	18.0	0.71
		F	21.3	15.9	28.0	18.4	23.3	15.8	
	22:4n-6	M	3.5	0.5	3.1	1.3	4.1	0.6	0.12
		F	3.6	0.5	2.4	1.2	3.7	0.5	
	22:5n-6	M	12.4	0.2	1.8	0.2	8.5	0.2	0.68
		F	21.6	0.3	4.1	0.2	16.9	0.4	
	22:5n-3	M	0.5	6.9	0.3	6.1	0.7	5.7	0.38
		F	0.5	6.5	0.2	3.9	0.3	3.8	
	22:6n-3	M	2.9	20.1	1.8	10.9	3.0	13.3	0.70
		F	3.1	23.9	3.5	17.8	4.1	20.9	

[a]Percentage of the fatty acids of each class; means of two rats. Other PUFA not shown (<1%): 18:3n-6, 20:2n-6, 20:3n-6, 18:3n-3, 20:5n-3.
[b]<0.1%.

DISCUSSION

The incorporation of dietary *trans* fatty acids, particularly of elaidic acid, in organ lipids has been studied extensively (4–12,14,22–30). Our results are in agreement with the general findings that most *trans* octadecenoic acids, including elaidic acid, readily incorporate into organ TG and in the 1-position of phosphoglycerides, where they substitute for saturated fatty acids. Known exceptions are brain lipids of post-weaning rats (4,5,29) testis phospholipids (4,5,25) and heart cardiolipin (30). This can be explained by the reduced utilization of exogenous fatty

TABLE 7

PUFA of Brain Lipids[a]

Lipid class	Fatty acid	Experimental groups: P	PL	E	EL	O	OL	Pooled SEM
PC	20:4n-6	5.6	4.9	5.9	5.6	5.8	5.3	0.11
	22:4n-6	0.9	0.6	1.1	0.7	1.0	0.6	0.03
	22:5n-6	1.8	—	1.6	—	1.8	—	0.08
	22:6n-3	1.4	3.5	1.5	4.0	1.5	3.7	0.11
	PUFA[b]	9.8	8.9	10.0	10.3	10.2	9.6	0.22
PE	20:4n-6	16.6	13.9	16.1	13.6	15.5	13.6	0.15
	22:4n-6	9.0	6.5	10.5	6.6	9.6	6.8	0.19
	22:5n-6	10.8	1.0	8.4	0.9	10.7	1.0	0.27
	22:6n-3	10.2	23.5	9.6	23.3	10.5	23.3	0.51
	PUFA	46.7	44.9	44.7	44.4	46.4	44.8	0.56
PS	20:4n-6	4.1	3.6	4.0	3.5	4.3	3.5	0.28
	22:4n-6	4.2	3.2	4.4	3.0	4.3	3.0	0.13
	22:5n-6	12.3	1.7	9.6	1.5	12.0	1.6	0.55
	22:6n-3	8.6	23.2	8.1	19.7	9.1	20.2	1.11
	PUFA	29.3	30.5	26.0	27.8	29.7	28.3	1.57

[a]Percentage of the fatty acids of each class; means of four rats (two males and two females). Other PUFA (<1%): 20:2n-9, 18:2n-6, 20:3n-6, 22:5n-3; 18:3n-3 and 20:5n-3 are absent of the three classes; there is no C22:5n-3 in PC.
[b]PUFA: Sum of the percentages of the four PUFA listed.

acids by brain and testis phospholipids and by the low saturated fatty acid content of CL. The presence of *trans*-octadecenyl chain in the plasmalogens of heart mitochondria, recently demonstrated by Wolff et al. (31), had not been looked for in this study but would deserve confirmation. In a previous work (14), the elaidic acid contents of organ lipids of rats fed elaidic acid were shown to be depressed when the dietary supply of linoleic acid was low. This possibly was caused by an increase of fatty acid oxidation, due to linoleic acid deficiency. This work shows that in linoleic acid-provided animals, the supply of linolenic acid does not influence the elaidic acid contents of tissue lipids.

Female rats are known to have higher levels of PUFA in the lipids of their organs than males (32). The observed sex differences were small in the brain, larger in the adrenals and very marked in the heart. The PUFA profiles, especially of heart PE, showed the ability of female rats over male rats to synthetize and/or to incorporate the end-products of the elongation-desaturation enzyme system: 22:5n-6 and 22:6n-3.

The effects of the dietary supply of linolenic acid on the PUFA profiles varied widely depending on the organ. These effects were remarkably low in testes where the main PUFA, 22:5n-6, was unchanged but were evident in adrenals, heart and brain, as previously observed (33,34). 22:6n-3 still was present in fairly high amounts in brain PE and PS of linolenic acid-deprived rats, illustrating the retention of this fatty acid by nervous tissue, even when the supply is very low (13). On the contrary, 22:5n-6 appears in high amounts in organ phospholipids only in rats that have been fed both high levels of linoleic acid and very low levels of linolenic acid (33-36).

Dietary elaidic acid depresses the levels of some PUFA in the lipid classes of several organs. Although the greatest effects were seen in the heart, the widespread distribution of the phenomenon in the body supports the hypothesis that elaidic acid acts on the biosynthesis of PUFA rather than on the incorporation in organ lipids. These changes are fairly complex: the biosynthesis of arachidonic acid is inhibited (as seen by the decrease of linoleic acid in the lipids of adrenals and heart) but the following steps of PUFA biosynthesis, elongation and −4 desaturation, through which 22:5n-6 and 22:6n-3 are biosynthetized, are most affected. This is more evident in the n-6 than in the n-3 EFA series, therefore in the linolenic acid-deprived rather than in the linolenic acid-fed rats. Moreover, this effect is of greater intensity in females than in males.

Diets containing partially hydrogenated fats have been shown to intensify EFA deficiency (4,6-9) and to induce a decrease of some PUFA contents in organ lipids (4,9,11,24,28,37). The *trans,trans* isomer of linoleic acid when fed to rats is a powerful inhibitor of the biosynthesis of n-6 and n-9 PUFA (38-41) and, more specifically, of the 6-desaturase (39,41,42) in either linoleate-deprived or linoleate-fed rats. Dietary elaidic acid lowers the arachidonic acid and 20:3n-9 contents of tissue phospholipids (blood, liver, heart) of linoleic acid-deficient rats, though to a lesser estent than the *trans,trans* 18:2 isomer (4,22, 28,43,44). In linoleic acid-provided animals, the feeding of elaidic acid causes an increase of linoleic acid and a decrease of 22:5n-6 in organ phospholipids; arachidonic acid is only slightly reduced (14,22). The 9-, 6- and 5-desaturases of rat liver microsomes are inhibited in vitro by a number of *cis*- and *trans* octadecenoic acid isomers, including elaidic acid (45,46). Recently, elaidic acid and other n-9, *trans* monoenes have been found to depress in vitro the 6- and 5-desaturase activities of human skin fibroblasts (47,48). In vivo effects of dietary fats containing trans monoenes are less clear (24,49,50): the decrease of 6-desaturase (49) or the increase of 5-desaturase (24) were not found by all authors. Effects of *trans* fatty acids on fatty acid elongation and 4-desaturation activities have not been directly observed yet.

Feeding partially hydrogenated soybean oil (PHSO) containing ca. 20% *trans* fatty acids to linoleic- and linolenic acid-provided rats, Lawson et al. (11) observed a reduction of the arachidonic acid contents of the phospholipids in all organs studied, mainly in the liver. The inhibition was specific to the n-6 EFA series; n-3 PUFA were either unchanged or increased by the dietary treatment. In feeding concentrates of *trans* or of *cis* 18:1 isomers from the same PHSO to rats, the same authors demonstrated that these effects were due to the *trans* isomers (12). Although the *trans* octadecanoic acids used by Lawson et al. were a complex mixture of positional isomers, their results and ours agree that the inhibition of PUFA biosynthesis produced by feeding *trans* monoenes can be observed in EFA-provided animals and is more marked on the n-6 PUFA series than on their n-3 homologues.

Further research is needed to elucidate the interactions of dietary fatty acid isomers with essential fatty acids and to evaluate the consequences for membrane, cell and organ function, particularly in the heart.

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Injection of Tridocosahexaenoyl-Glycerol Emulsion and Fatty Acid Composition of Blood Cells

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An injectable emulsion of docosahexaenoic acid (DHA) was prepared. One hundred ml of the emulsion contained 3 g of 93%-pure 1,2,3-tridocosahexaenoylglycerol (DHA-TG), 1.2 g of 93%-pure 2-docosahexaenoyl-phosphatidylcholine as an emulsifier and 2.5 g of glycerol. Thirty ml of the emulsion of DHA-TG was injected into three rabbits on days 1 and 4 of the study. Blood was taken on day 0, on day 4 just before the second injection and on day 7. The percentage of DHA in the total phospholipid fraction of platelets increased from 0.46% (day 0) to 1.88% (day 4, $p < 0.05$) and 3.66% (day 7; $p < 0.02$ vs day 0). The percentage of eicosapentaenoic acid (EPA) increased from 0.46% (day 0) to 1.03% (day 4, $p < 0.02$) and 1.63% (day 7: $p < 0.05$ vs day 0). The percentage of arachidonic acid (AA) decreased from 9.45% (day 0) to 4.31% (day 4, $p < 0.05$) and 6.68% (day 7; $p < 0.02$ vs day 0). The percentage of DHA in the total phospholipid fraction of erythrocyte membranes increased from 0.23% (day 0) to 0.91% (day 4, $p < 0.05$) and 1.52% (day 7; $p < 0.005$ vs day 0); that of EPA increased from 0.21% (day 0) to 0.34% (day 4, $p < 0.005$%) and 0.52% (day 7, $p < 0.01$ vs day 0); that of AA was unchanged. Blood lipids were the same before and after the two injections of the emulsion, except that free fatty acids decreased markedly from 0.32 to 0.06 mEq/l ($p < 0.02$). On day 8, free AA (2 mg/kg) was injected into ear veins of the three treated rabbits and of four control rabbits (not treated with DHA-TG). All the control rabbits died a few minutes after the AA injection, but none of the DHA-treated rabbits died after AA injection ($p < 0.01$). An emulsion of DHA-TG may be useful for patients having immediate risk of thrombosis or for those who need DHA but cannot take it orally.
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It has long been known that ω6 fatty acids are essential to health (1), but it is only fairly recently that several studies have suggested that ω3 fatty acids also are important for normal tissue functions (2–5). Among them, docosahexaenoic acid (DHA) seems to be very important for the nervous system including the retina, since DHA is the major fatty acid in the phospholipid fraction in the gray matter of the brain (6) and in the retina (7). Deprivation of ω3 fatty acids from the diet for two generations could inhibit the development of functionally normal retinas in monkeys (5). Also, it is reported that a disturbance of the peripheral nervous system developed in a 6-year-old girl after long-term, total parenteral feeding of a diet that contained little ω3 fatty acid (3).

Although α-linolenic acid (18:3ω3), whether ingested or administered parenterally, could be converted to DHA, the rate of conversion is low (8,9). Therefore, we thought it was important to develop a DHA emulsion for people who need DHA but cannot take it orally. The purpose of this investigation was to observe how rapidly a DHA emulsion injected into rabbits could increase the DHA levels in blood cells.

MATERIALS AND METHODS

Experimental design. Three rabbits were used, each weighing ca. 3 kg. Throughout the experiment, they were fed a standard rabbit diet (RM-3, Funabashi Farms, Chiba, Japan) (Table 1), the major lipid component of which was soybean oil. Blood was taken from ear veins on days 0, 4 and 7. Thirty ml of 1,2,3-tridocosahexaenoyl-glycerol (DHA-TG) emulsion was injected via the ear veins on day 1, and on day 4 just after the second blood sampling. On day 8, the three rabbits were injected through ear veins with 6 mg of arachidonic acid (AA; Sigma Chemical Co., St. Louis, MO) dissolved in 100 mM sodium bicarbonate. Four control rabbits, each weighing about 3 kg and not treated with the DHA-TG emulsion, were injected with AA in the same manner.

Preparation of emulsion. Sardine oil was extracted from edible parts of sardines, deacidified and decolorized. After separation of saturated lipids by crystallization at −70 C, a polyunsaturated fatty acid–rich triglyceride mixture was saponified and then methylated

TABLE 1

Composition of Rabbit Diet RM-3 (Wt%)[a]

Crude protein	15.8%
Crude lipids	4.0%[b]
Crude fiber	13.3%
Crude mineral	9.6%
Soluble material containing no nitrogen	50.3%
Metabolic energy	3.6 kcal/g

[a]According to the manufacturer's brochure; a vitamin mixture is also added.
[b]Lipids constitute 10 cal% and consist mostly of soybean oil.

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Abbreviations: DHA, docosahexaenoic acid; DHA-TG, 1,2,3-tridocosahexaenoyl-glycerol; AA, arachidonic acid; DHA-PC, 2-docosahexaenoyl-phosphatidylcholine; PRP, platelet-rich plasma; EPA, eicosapentaenoic acid; RBC, red blood cell; EPA-TG, 1,2,3-trieicosapentaenoyl-glycerol.

TABLE 2

Fatty Acid Composition (Mol%) of Lipids Used

	Lipids		
		2-DHA-PC	
Fatty acids	DHA-TG	Position 1	Position 2
16:0		26	
18:0		7	
18:1		20	
18:2		45	
18:3ω3		2	
20:4ω6	1		1
22:4	1		1
22:5ω3	2		2
22:6ω3	93		93

One hundred ml of emulsion contained 10 g of DHA-TG, 1.2 g of DHA-PC and 2.5 g of glycerol.

TABLE 3

Changes in Some Blood Lipids and Enzymes by 2 Infusions of 30 ml DHA-TG Emulsion into Rabbits

Parameters	Day 0	Day 7
GOT (U)	26 ± 17	16 ± 3
GPT (U)	21 ± 8	22 ± 4
γ-GTP (IU)	3 ± 1	2 ± 1
Total cholesterol (mg/dl)	39 ± 16	42 ± 7
Triglycerides (mg/dl)	28 ± 4	27 ± 6
Free fatty acids (mEq/l)	0.32 ± 0.04	0.06 ± 0.004[a]

Serum of days 0 and 7 was analyzed. GOT, glutamic-oxaloacetic transaminase; GPT, glutamic-pyruvic transminase; γ-GTP, γ-glutamyl transpeptidase.

[a]$p < 0.02$.

(10). Methyl esters of polyunsaturated fatty acids were separated to obtain a DHA methyl ester fraction by high pressure liquid chromatography (11). One tenth mol of glycerol and 0.3 mol of free DHA, which was obtained by hydrolysis of DHA methyl esters, were reacted in dimethyl sulfoxide with 0.3 mol of N,N′-carbonyl-diimidazole as a condenser and imidazole sodium salt as a catalyst. After overnight reaction at room temperature, DHA-TG was extracted by hexane and purified by a silica gel column (12). α-Tocopherol (0.2%) was added to DHA-TG. 2-Docosahexaenoyl-phosphatidylcholine (DHA-PC) was synthesized as follows: 0.1 mol of lysophosphatidylcholine, which was enzymatically prepared from soybean oil phosphatidylcholine with phospholipase A_2, and 0.1 mol of free DHA were reacted in chloroform with 0.1 mol of N,N′-carbonyl-diimidazole and imidazole sodium salt as a catalyst for 1 hr at room temperature. DHA-PC was extracted by chloroform/methanol (2:1, v/v) and purified by a silica gel column. DHA-TG was emulsified with DHA-PC according to the method of Geyer et al. (13). One hundred ml of the DHA-TG emulsion contained 10 g of DHA-TG, 1.2 g of DHA-PC and 2.5 g of glycerol. The fatty acid composition of the lipids used in the experiments is shown in Table 2.

Analysis of fatty acid composition. The fatty acid composition in total phospholipids in platelets was analyzed as follows. Platelet-rich plasma (PRP) was prepared by centrifugation of blood citrated with 1/10 volume of 3.8% sodium citrate. Platelets from 0.4 ml of PRP were washed with 0.9% saline, reconstituted in 0.4 ml of saline and sonicated for 1 min. Total lipids were extracted with 10ml of chloroform/methanol (2:1, v/v) The phospholipid fraction was obtained as the unmoved fraction following thin layer chromatography on silica gel plates using petroleum ether/ether/acetic acid (60:30:1, v/v/v) as solvent. Methyl esters of fatty acids of total phospholipids were prepared with boron trifluoride and methanol (14). A glass capillary column, Advans-DS, 0.24 mm × 25 m (Shinwakagaku, Kyoto), was used to analyze the fatty acid methyl esters. The column was attached to a GC7A gas chromatograph (Shimadzu, Tokyo). The injection was at 250 C; separation with N_2 gas as a carrier (40 ml/min) was isothermal at 200 C; detection was by flame ionization. Erythrocytes were separated from citrated blood, washed and hemolyzed by osmotic shock. The membrane fraction was separated by centrifugation and washed twice. The fatty acid analysis of phospholipids of membranes was then performed as described above.

Blood chemistry, platelet aggregation and statistics. PRP was prepared as described above. Platelet aggregation was measured with an aggregometer (Model PAT-4M, Niko Bioscience, Tokyo), adding 20 μl of 50 μM ADP (Sigma), 100 μg/ml of collagen (Hormon-Chemie, Munich, FRG) or 100 μM AA as an aggregant to 180 μl of PRP to make up a 200-μl mixture. Serum total cholesterol (15), triglycerides (16) and free fatty acids (17) were measured enzymatically; glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase and γ-glutamyl transpeptidase were measured spectrophotometrically with an autoanalyzer. Data were expressed as means ± SD. Student's t test or χ^2 test was used for data evaluation.

RESULTS

There were no significant differences in body weight between days 0 and 7 in the three rabbits. Changes in blood chemistry are shown in Table 3. There was a significant reduction of free fatty acids following the two injections of DHA emulsion. Changes in the fatty acid composition of total phospholipids of platelets and red blood cell (RBC) membranes are shown in Tables 4 and 5, respectively. In both platelets and RBC membranes, DHA and eicosapentaenoic acid (EPA) levels were increased significantly. There were no significant differences in platelet aggregation induced by any aggregants used in the experiments between days 0 and 7. However, the injection of AA (2 mg/kg) into ear veins on day 8 caused death in none of the three rabbits, while the same injection killed all four control rabbits, which had been on the same diet and were not treated with DHA emulsion ($p < 0.01$).

TABLE 4

Changes in Fatty Acid Composition of Platelet Total Phospholipids[a]

Fatty acids	Fatty acid composition (mol %)		
	Day 0	Day 4	Day 7
16:0	24.2 ± 1.3	31.3 ± 2.4^{b}	22.4 ± 0.4^{b}
18:0	27.4 ± 0.4	23.3 ± 2.0	26.8 ± 1.0
18:1	8.3 ± 0.2	10.0 ± 1.0	8.4 ± 0.6
18:2ω6	19.9 ± 2.0	14.6 ± 3.4	22.2 ± 0.7
18:3ω3	0.61 ± 0.10	0.56 ± 0.11	0.76 ± 0.04
20:4ω6	$9.45 \pm 0.39^{b,c}$	$4.31 \pm 0.80^{b},^{b'}$	$6.68 \pm 0.27^{b',c}$
20:5ω3	$0.46 \pm 0.07^{b,c}$	1.03 ± 0.16^{c}	1.63 ± 0.32^{b}
22:6ω3	$0.46 \pm 0.10^{b,c}$	1.88 ± 0.42^{b}	3.66 ± 0.53^{c}

[a]Thirty ml of DHA emulsion was injected on day 1 and day 4 just after the second blood sampling. Values with the same superscript are significantly different from each other. b(b'), $p < 0.05$; c, $p < 0.02$.

TABLE 5

Changes in Fatty Acid Composition of Phospholipids in RBC Membranes[a]

Fatty acids	Fatty acid composition (mol %)		
	Day 0	Day 4	Day 7
16:0	27.5 ± 0.7	28.3 ± 0.4	28.0 ± 0.5
18:0	18.1 ± 0.1	17.9 ± 0.4	17.7 ± 0.3
18:1	$12.5 \pm 0.6^{b,c}$	11.6 ± 0.5^{b}	11.2 ± 0.4^{c}
18:2ω6	31.0 ± 0.0	30.8 ± 0.5	30.8 ± 0.4
18:3ω3	1.21 ± 0.09	1.31 ± 0.09	1.29 ± 0.03
20:4ω6	3.46 ± 0.10	3.44 ± 0.05	3.49 ± 0.04
20:5ω3	$0.21 \pm 0.04^{d,e}$	$0.34 \pm 0.04^{c,e}$	$0.52 \pm 0.07^{c,d}$
22:6ω3	$0.23 \pm 0.05^{b,e}$	$0.91 \pm 0.12^{b,b'}$	$1.52 \pm 0.07^{b',e}$

[a]Thrity ml of DHA emulsion were injected on day 1 and day 4 just after the second blood sampling. Values with the same superscript are significantly different from each other. b(b'), $p < 0.05$; $p < 0.02$; d, $p < 0.01$; e, $p < 0.005$.

DISCUSSION

Almost all the papers dealing with ω3 fatty acids as nutritional components or as drugs have described their oral administration. If one considers the importance of ω3 fatty acids from the standpoint of long-term nutrition, oral administration would appear to be the only feasible means of administration. However, if one considers the pharmacological aspects of ω3 fatty acids, it may sometimes be important to administer them in a manner such that their concentration in the blood can be raised quickly. For immature babies or babies under 6 months of age whose gastrointestinal system does not function, the injection of DHA becomes extremely important because brain nerve cells, the membranes of which contain an enormous amount of DHA, still proliferate during the first six months of life. Indeed, the huge demand of neonatals for DHA is emphasized by the estimate that in the brain tissue up to 50% of the ultimate mass of DHA accumulates after birth (18).

It is only recently that a few papers have described the intravascular administration of lipid emulsions containing α-linolenic acid for the supplementation of ω3 fatty acids. However, the conversion rate of α-linolenic acid to DHA is not very high, and the increment in DHA in tissues may take a long time (9,19). We have already developed an injectable emuslion of 1,2,3-trieicosapentaenoyl-glycerol (EPA-TG) and described the effect of this emulsion on the fatty acid composition in blood (20). Although the injection of EPA-TG into rabbits increased EPA levels in blood, it did not increase DHA levels in blood. Therefore, if patients who cannot eat anything at all need DHA, whether as a supplement or for pharmacological purposes, an emulsion containing DHA is indispensable. It is interesting to note that the injection of DHA-TG emulsion increased not only DHA levels but also EPA levels both in platelets (Table 4) and in RBC membranes (Table 5). The retroconversion of orally administered DHA has already been shown (21). In this study, the retroconversion was shown to occur without any absorption process of DHA in the intestine.

With regard to the effects of blood chemistry of this emulsion, we did not observe any remarkable changes except for a significant decrease in free fatty acid levels in serum after the injection of the DHA-TG emulsion. In our previous study (20), we showed that the levels of serum free fatty acids were not changed significantly by the injections of EPA emulsion (0.31 ± 0.20 to 0.41 ± 0.10 mEq/1), but were increased significantly by injections of soybean oil emulsion (0.32 ± 0.10 to 0.56 ± 0.10 mEq/1, $p < 0.05$) in rabbits. Although the hypolipidemic effect of fish oil is very well investigated (22), only a few studies report the effect of administration of pure or concentrated DHA on enzyme activities related to lipid metabolism (23,24). Iritani et al. reported that the administration of a mixture of docosapentaenoic acid methyl ester and DHA methyl ester decreases fatty acid synthetase activity in the rat liver (23). Morisaki et al. reported that the activities of acyl-CoA synthetase are not changed in the rat aorta by DHA administration (24). Therefore, the depressive effect of DHA emulsion on serum free fatty acids may be mediated at least partially by decrease in fatty acid synthesis. However, the available data are so limited that the mechanism of action of DHA on serum free fatty acids is not clear and remains to be elucidated.

DHA is known to depress platelet aggregation after oral administration (21). However, we did not observe a significant depression of platelet aggregation. This may be due to the small number of experimental animals in the present study.

Talesnik infused free DHA to isolated platelet-free rat heart and observed a protective effect of DHA against AA-induced coronary flow reduction (25). He suggested that the incorporation of DHA by the isolated heart would inhibit the cyclooxygenase (26) in the coronary vessel walls, interfering with the generation of vasoconstrictive metabolites from AA (probably thromboxane) (25). Because we could not observe an inhibition of AA-induced platelet aggregation by the infusion of DHA emulsion, the protective effect of DHA against

the AA-induced thrombosis model shown in the present investigation may be mediated through the inhibition in the vessel walls of cyclooxygenase activity, as suggested by Talesnik.

In conclusion, a DHA-TG emulsion may be useful for patients with immediate risk of thrombosis or for those who need DHA but cannot take it orally.

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Effect of Marginal Zinc Deficiency on the Apolipoprotein-B Content and Size of Mesenteric Lymph Chylomicrons in Adult Rats[1]

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To investigate the mechanisms underlining the impaired intestinal absorption of lipids in zinc deficiency, the apo-B content and chemical composition of chylomicrons from marginally zinc-deficient rats fed 2.8 ppm of dietary zinc (ZD) were compared with those from pair-fed (PF) and ad libitum control (CT) groups fed an adequate level (30.8 ppm) of zinc. Chylomicrons, obtained by cannulating the mesenteric lymph, were isolated by ultracentrifugation at 1.3×10^6 g/min at 12 C and purified by 2% agarose column chromatography. Apolipoprotein- (apo) B was separated by the method of isopropanol precipitation. The apo-B concentration of chylomicrons was lowered significantly in ZD group. The apo-B contents of chylomicrons in ZD, PF and CT rats, as expressed as % chylomicron protein, were 8.7 ± 0.1, 11.5 ± 0.5 and 10.7 ± 0.7%, respectively. No significant differences were noted between ZD and PF groups in total protein (TP), phospholipid (PL), triglyceride (TG) and cholesterol (CH), although there was a slight decrease in TG and an increase in CH in CT rats compared with ZD and PF groups. The ratio of the core to surface constituents, as determined by TG/(TP + PL), was significantly higher in ZD group relative to the controls, suggesting that chylomicrons from ZD rats were larger. This finding was consistent with the appearance of larger chylomicron particles in the lacteal of the intestinal mucosa following lipid ingestion. These findings suggest that the intestinal synthesis of apo-B may be defective in zinc-deficient rats and may explain in part the impaired absorption of dietary lipids observed in zinc deficiency.

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Possible defects in the intestinal formation of chylomicrons in zinc deficiency was first demonstrated by Koo and Turk (1). In advanced zinc deficiency in young male rats (1), lipid droplets of abnormally large sizes were shown to accumulate in the intestinal mucosa following oral ingestion of triglyceride; this impaired the intestinal transport of mucosal lipids into the circulation. Consistent with this observation, the intestinal absorptive epithelium exhibited marked decreases in the granular endoplasminc reticulum and Golgi complex (2), which are the major sites for the synthesis of chylomicrons (3) and packaging of lipids (4) during mucosal lipid transport. Our recent study (5) indicated that even in marginally zinc-deficient rats, the lipid droplets in the absorptive epithelium displayed a strong tendency to coalesce into larger droplets. The composition of the soluble apolipoproteins of chylomicrons obtained from the mesenteric lymph of these rats was shown to be altered markedly.

However, whether the distribution of apo-B in chylomicrons is affected by zinc deficiency is not known. Apo-B is a major apolipoprotein synthesized in the intestine that is believed essential for chylomicron formation. This experiment was conducted to determine the apo-B content of chylomicrons and to examine further the relationship between the composition and size of chylomicrons appearing in the lacteal, as affected by a marginal level of zinc deficiency.

MATERIALS AND METHODS

Animals and diets. Male Fischer rats (CDF[F-344]CrlBR; Charles River Breeding Lab., Inc., Wilmington, MA) were placed individually in plastic cages with stainless-steel wire bottoms in a windowless room and were subjected to a light cycle with the light period from 1400 to 0200 and dark period from 0200 to 1400. Temperature and humidity of the room were maintained at 23 C and 55–60%, respectively. The rats were acclimatized for one week and fed a commercial rat chow (Wayne Laboratory Animal Diets, Denver, CO) during this period. The rats with the mean weight of 138 ± 5 g were divided into three groups: 1) a zinc-deficient (ZD) group fed ad libitum a basal diet supplemented with 2.0 ppm of zinc as the carbonate. The basal diet, as described in Table 1, contained 0.8 ppm of zinc and the total zinc concentration of the zinc-deficient diet was 2.8 ppm; 2) a pair-fed group (PF), which was matched closely with the ZD group in respect to the body weight at the start of dietary treatment and were fed the basal diet supplemented with 30 ppm of zinc. The rats in this group were fed daily the same amounts

TABLE 1

Composition of Basal Diet[a]

Ingredient	% Diet
Egg white solids	20.0
DL-Methionine	0.3
Sucrose, granular	36.0
Corn starch	30.9
Corn oil	4.0
Cholesterol, USP	1.0
Cellulose, powder	3.0
Choline chloride (Cholfeed, 50%)	0.3
Vitamin mix[b]	1.0
Mineral mix[c]	3.5

[a]Purchased from Zeigler Brothers, Inc. (Gardeners, PA).
[b]According to the recommendations of the American Institute of Nutrition (AIN), except that the concentration of biotin in the mix was increased to 50 mg/kg (ref. 15).
[c]According to the AIN recommendations but with no zinc. The basal diet formulated with the above ingredients contained 0.8 ppm of zinc, as determined by atomic absorption spectrophotometry.

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Abbreviations: CH, cholesterol; CT, ad-libitum control; HDL, high density lipoproteins; PF, pair-fed; PL, phospholipid; TG, triglyceride; TMU, tetramethylurea; TP, total protein; ZD, zinc deficient.

of diet as consumed by ZD rats and 3) another control group was fed the above zinc-adequate diet ad libitum. All rats were given distilled-deionized water via a stainless-steel water delivery system.

Cannulation of lymph duct. The cannulation of the mesenteric lymph duct was performed by a modification of the method of Warshaw (6); the modified procedure has been described in detail in previous studies (5,7). After 10–12 weeks of dietary treatment, the rats were anesthetized with diethylether. With a subcostal incision, the mesenteric lymph ducts were exposed, and the minor duct was cut and sealed with cyanoacrylate glue. The major lymph duct was cannulated with polyethylene tubing (PE-50, Clay Adams, Parsippany, NJ) coated with TDMAC-heparin complex (Polysciences, Inc., Warrington, PA). An indwelling catheter (PE-90, Clay Adams) was placed via the mouth into the pylorusduodenal junction and secured by a ligature. Immediately after closing the incision with suture (4-0 silk, Ethicon Inc., Somerville, NJ), the rats were placed in restraining cages (8) and infused by an infusion pump (Harvard Apparatus, Model 935, South Natick, MA) via the duodenal catheter with a glucose saline (5% glucose, 0.87% NaCl and 0.03% KCl) at the rate of 2.0 ml/hr for 18 to 20 hrs (overnight). During the postoperative recovery, the room temperature was maintained at 27 to 29 C by using an electric heater.

Collection of lymph and isolation of chylomicrons. After the postoperative recovery, the rats were infused with a lipid emulsion via the duodenal catheter. The infusion rate was increased to 3.0 ml/hr to increase the flow and volume of lymph. The lipid emulsion consisted of a 1:1 (v/v) mixture of Intralipid® (10% soybean oil, 1.2% phospholipid and 2.25% glycerin, USP; KabiVitrum, Inc., Alameda, CA) and a glucose saline (5% glucose, 0.87% NaCl and 0.03% KCl). Lymph was collected via the cannula into conical plastic tubes cooled in an ice bath for eight hr and defibrinated by filtration of glass wool. The average volume of defibrinated lymph collected per rat was 10.5 ± 0.9 ml. The filtrate was overlayered with 150 mM NaCl solution containing 0.04% Na_2EDTA, pH 7.4, in polyallomer tubes and centrifuged at $1.3 \times 10^6 \times$ g/min at 12 C using a 50.3 Ti rotor in a Beckman L5-75B Ultracentrifuge (Spinco Division, Palo Alto, CA). The packed top fraction of chylomicrons was collected by slicing the tubes and were purified by a single passage through 2% agarose column (Biogel A-50, 100–200 mesh, Bio-rad Labs., Richmond, CA) according to the method of Sata et al. (9). The column was eluted at 12 C with 150 mM NaCl containing 0.04% Na_2EDTA and 0.02% sodium azide, pH 7.0. Elution of chylomicrons was monitored by absorbance at 280 nm (LKB 2089 Uvicord III, LKB Instruments, Rockville, MD). The column fractions of chylomicrons were combined and concentrated by centrifugation as above.

Determination of chylomicron apo B. The apo-B content of chylomicrons was measured by the method of isopropanol precipitation as described by Egusa et al. (10). One to 1.5 ml of chylomicron suspension in 150 mM NaCl was mixed vigorously with an equal volume of 100% isopropanol (A-416, Fisher Scientific Co.-certified ACS, Fair Lawn, NJ) and incubated at room temperature for 18–20 hr. The mixture was centrifuged at 1500 × g for 60 min, and the supernatant was discarded. The precipitate was washed twice with 2.0 ml of 50% isopropanol and centrifuged as above. The precipitate was dispersed in 100% isopropanol for two hr and recovered by centrifugation as above. Then it was solubilized by incubation in 50 μl of 1N NaOH at 37 C, and the protein (apo-B) content was determined by the method of Kashyap et al. (11).

Analysis of chylomicron protein and lipids. The concentration of total protein was determined by a modified Lowry method after washing purified chylomicrons twice with chloroform (11). The concentrations of triglyceride and cholesterol were determined by the methods of Rudel and Morris (12) and of Neri and Frings (13), respectively. Phospholipid was determined by the method of Raheja et al. (14).

Electron microscopy of chylomicrons in the lacteal. Rats were fasted for 18 hr and fed 1.0 ml of corn oil by gastric intubation. Two hours later, the rats were killed by cervical dislocation, and 1-cm jejunal segments anterior to the ligament of Treitz were removed and immediately fixed in ice-cold 3.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for one hr. The fixed tissues were minced into 1–2 mm blocks, fixed in glutaraldehyde at room temperature for 1.5 hr and postfixed in 1% osmium tetraoxide. The tissue blocks were dehydrated in ethanol and propylene oxide and embedded in Epon 812. The ultrathin cross sections of the jejunal villi were cut and stained with uranylacetate and lead citrate. The sections were examined using an electron microscope (Phillips EM 400, Phillips Electronic Instruments, Inc., Mahwah, NJ). The lacteals located below the basement membranes of the villi were photographed at 7,000 magnification and enlarged 2.5 times for determination of chylomicron sizes. The lacteal lumen that displayed well-dispersed chylomicron particles were selected. The diameters of approximately 1,000 chylomicron particles from four micrographic fields were measured in each group, and the size distributions of the particles were compared statistically.

Plasma zinc analysis. After fasting rats for 18 hours, blood samples were withdrawn from the orbital sinus at the mid-dark phase (0800 to 0900) of the light cycle. Serum zinc was determined by atomic absorption spectrophotometry (Perkin-Elmer Co., Norwalk, CT). The zinc standards were prepared from a Fisher-certified reference standard (Fisher Scientific Co., Fair Lawn, NJ).

Statistics. Analyses of variance and Duncan's multiple range test were used for comparisons among the three group means. The statistical analyses were performed by using a computer software (PC ANOVA, Human Systems Dynamics, Northridge, CA). All data were expressed as mean ± SEM, and the level of significance was determined at $p < 0.05$, unless otherwise stated.

RESULTS

General observations. The food intake, body weight and serum zinc status of the rats, as affected by experimental diets, are shown in Table 2. In order to produce a marginal level of zinc deficiency, the rats in ZD group were fed a diet containing 2.8 ppm of zinc. This level of dietary zinc prevented a drastic decrease in diet consumption and resultant weight loss, as normally observed with extremely low levels of zinc. A significant reduction in food intake in ZD rats was noticeable only after seven weeks. The average body weight of ZD group at the end

of the experiment differed significantly from that of the ad-libitum control (CT) group but not from that of PF group. The serum level of zinc was significantly lower in ZD rats as compared with PF and CT controls but no external signs of zinc deficiency such as alopecia and skin lesions were visible at this stage of zinc deficiency.

The apo-B content and composition of lymph chylomicrons. The apo-B concentrations of lymph chylomicrons are shown in Table 3. The apo-B contents of chylomicrons from ZD, PF and CT groups were 0.25 ± 0.02, 0.41 ± 0.04 and 0.47 ± 0.07 μg per mg chylomicrons, respectively. The apo-B content in ZD group was significantly lower as compared with the PF and CT controls. A similar difference also was noted when expressed in terms of μg per mg chylomicron triglyceride. Apo-B represented 8.7% ± 0.1% of the total apoprotein content of chylomicrons in ZD group as compared with 11.5 ± 0.5 and 10.7 ± 0.7% in PF and CT groups, respectively (Table 3). Table 4 compares the protein and lipid composition of chylomicrons as affected by experimental diets. The percent concentrations of total protein and phospholipid of chylomicrons tended to decrease in ZD groups as compared with PF and CT groups. The dfferences between ZD and the two control groups were not significant statistically. Chylomicrons from CT group were significantly higher in cholesterol but slightly lower in triglyceride content as compared with those obtained from ZD and PF groups. There were no differences in the ratio of triglyceride (TG) to phospholipid (PL) among the three groups.

Size distribution of chylomicrons. In all groups, chylomicrons appearing in the lacteals were much smaller in size than the lipid droplets present in the cytoplasm of the absorptive epithelium. Figure 1 shows the morphological appearance and size distribution of chylomicrons found in the lacteal lumen during lipid transport. The average diameters of chylomicrons from ZD, PF and CT groups were 0.20, 0.13 and 0.14 μm, respectively. Approximately 78% of the chylomicron particles of ZD rats measured between 0.06 and 0.23 μm in diameter. A significant proportion (22%) of the particles was larger than 0.30 μm, with 5.5% of the total exceeding 0.5 μm. In contrast, most (>93%) of the particles of PF and CT controls were smaller than 0.23 μm in diameter, and the rest (<7%) ranged from 0.30 to 0.46 μm.

TABLE 2

Effect of Experimental Diets on the Food Intake, Body Weight and Serum Zinc Status of the Rats[a]

Group	Food intake (g/rat/day)		Body weight (g/rat)		Serum zinc (μg/ml)
	Initial	Final	Initial	Final	
ZD	15 ± 1	11 ± 1[a]	139 ± 5	236 ± 8[a]	1.26 ± 0.12[a]
PF	15 ± 1	11 ± 1[a]	136 ± 5	256 ± 9[ab]	1.89 ± 0.16[b]
CT	15 ± 1	14 ± 1[b]	140 ± 6	272 ± 13[b]	1.86 ± 0.04[b]

[a]Mean ± SEM of five rats. Means not sharing a common superscript within the same column are significantly different ($p < 0.05$).

TABLE 3

Effect of Experimental Diets on the Apo-B Content of Lymph Chylomicrons[a]

Group	Apoprotein-B content		
	μg/mg Chylomicron	μg/mg TG[b]	% Total protein
ZD	0.25 ± 0.02[a]	0.26 ± 0.02[a]	8.7 ± 0.1[a]
PF	0.41 ± 0.04[b]	0.44 ± 0.04[b]	11.5 ± 0.5[b]
CT	0.47 ± 0.07[b]	0.48 ± 0.08[b]	10.7 ± 0.7[b]

[a]Mean ± SEM of five rats. Means not sharing a common superscript within the same column are significantly different ($p < 0.05$).
[b]TG, triglyceride.

DISCUSSION

The gastrointestinal manifestations of acrodermatitis enteropathica, a hereditary human zinc-deficiency disorder, include chronic diarrhea and malabsorption of lipids (16). Lipid malabsorption in zinc deficiency has been well-demonstrated in previous studies using growing and adult rats (1,7). However, the mechanisms underlying the impaired intestinal absorption of lipids remain to be elucidated. In zinc-deficient young rats given an oral dose of triglyceride, abnormally large sizes of lipid droplets were shown to accumulate in the apical cytoplasm of the intestinal epithelium. Such characteristics of cytoplasmic lipid droplets were also demonstrated in marginally zinc-deficient adult rats in the absence of the externally

TABLE 4

The Protein and Lipid Composition of Lymph Chylomicrons[a]

Group	% Chylomicron)				
	Protein	CH[b]	TG[b]	PL[b]	TG/PL
ZD	0.28 ± 0.02	0.96 ± 0.20[a]	94.9 ± 0.4[a]	3.89 ± 0.27	24.4 ± 1.7
PF	0.39 ± 0.05	1.15 ± 0.28[a]	94.1 ± 0.3[a]	4.63 ± 0.26	20.3 ± 1.1
CT	0.40 ± 0.05	1.96 ± 0.30[b]	92.8 ± 0.4[b]	4.82 ± 0.24	19.3 ± 1.1

[a]Mean ± SEM of five rats. Means not sharing a common superscript within the same column are significantly different ($p < 0.05$).
[b]TG, triglyceride; PL, phospholipid; CH, cholesterol.

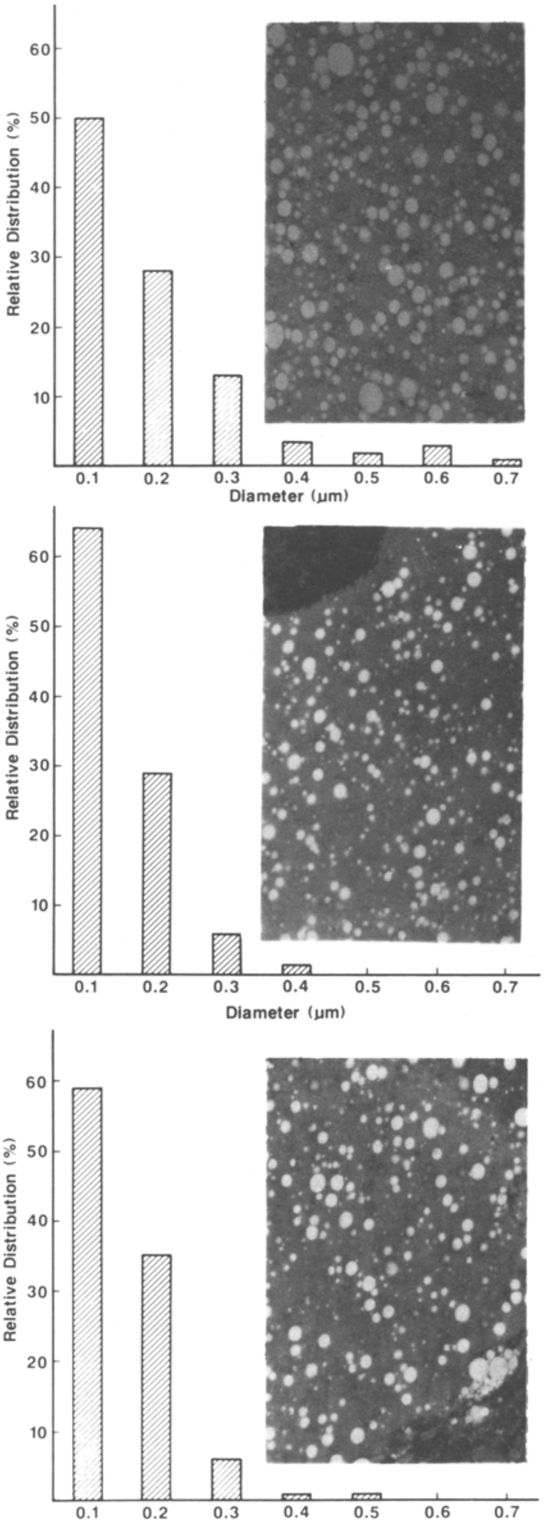

FIG. 1. The size distribution of chylomicrons appearing in the lacteal lumen of the intestinal villi. A significant proportion (22%) of chylomicrons was larger than 0.30 μm and 5.5% of the chylomicron particles exceeded 0.5 μm in diameter in ZD group (top). Most (>93%) of the particles of PF and CT controls were smaller than 0.23 μm in diameter. Chylomicrons greater than 0.5 μm in diameter were not found in the lacteals of the PF and CT controls. The marker bar = 0.5 μm.

manifested signs of zinc deficiency (5). However, specific molecular defects in chylomicrons produced by zinc deficiency currently are not known.

This study provides evidence for the first time that the apo-B concentration of lymph chylomicrons is lowered significantly in even a marginal (or subclinical) stage of zinc deficiency. The apo-B content of chylomicrons from zinc-deficient rats was decreased to 53 to 61% of that of PF and CT controls when compared on the basis of apo-B content per mg of chylomicron mass. The apo-B values for the control rats, as determined by isopropanol precipitation in the present study, are in excellent agreement with those obtained by the method of tetramethylurea (TMU) precipitation (17).

The importance of the protein moiety of chylomicrons in their formation and release into the lymphatics was first demonstrated by Sabesin and Isselbacher (18) in a study using rats treated with a protein-synthesis inhibitor. Further evidence for the impaired chylomicron formation and lipid transport during protein-synthesis inhibition has been provided in subsequent studies (19–22), although controversy still exists concerning the obligatory role of protein synthesis in the release of chylomicrons (23).

In the earlier study (5), we observed that the lipid droplets accumulating in the epithelial cytoplasm of zinc-deficient rats frequently were larger than 5 μm in diameter. Present data showed no evidence for the entry of such large chylomicrons in the lacteal (Fig. 1), suggesting the inability of those lipid droplets (or chylomicrons) to exit into the lymphatic system. Nevertheless, the average diameter of chylomicron particles appearing in the lacteal of zinc-deficient rats still was significantly larger than that of pair-fed and ad libitum controls. Approximately 22% of the chylomicrons from zinc-deficient rats was larger than 0.3 μm in diameter, and 5.5% exceeded 0.5 μm, whereas the majority (93%) of chylomicrons from pair-fed and ad libitum control rats was smaller than 0.23 μm, with ca. 7% ranging between 0.30 to 0.46 μm. A similar increase in the size of lymph chylomicrons also was observed in the rats treated with a protein-synthesis inhibitor (acetoxycycloheximide) (21).

Previously, it has been postulated that the defective synthesis of apo-B in the intestinal epithelium may be responsible for such an increase in chylomicron size and cytoplasmic accumulation of lipid droplets during inhibition of protein synthesis (18). Because of the non-specific nature of the protein-synthesis inhibitors, whether such a phenomenon is due specifically to the lack of intestinal apo-B or other apolipoproteins remains debatable. Glickman et al. (24) showed that in acetoxycycloheximide-treated rats both apo-B and apo-A-I were reduced markedly in the intestinal mucosa during lipid absorption. A significant decline in chylomicron apo-A-I was demonstrated in rats injected with acetoxycycloheximide (22). In a previous study (5), we observed that a marginal level of zinc deficiency produces significant decreases in the relative contents of apo-C and apo-E of lymph chylomicrons. Evidence indicates that chylomicrons are released into the circulation despite the deficiency of apo-C (25). Also, the data obtained from tracer studies (26,27) suggest that apo-E is not incorporated into intestinal nascent chylomicrons or very low density lipoproteins during their synthesis in the intestine and that it may not

be an essential component of nascent intestinal lipoproteins for their release into the lymphatics. These apolipoproteins mostly are acquired by nascent chylomicrons from plasma HDL upon their release into the lymphatics and blood plasma (28–31). Therefore, the decreases in chylomicron apo-C and apo-E in zinc-deficient rats (5) may reflect largely the decrease in the circulating level of HDL as previously demonstrated in zinc-deficient rats (32). Our recent data (33) have shown that the relative proportions of apo-E and apo-C of plasma high density lipoprotein (HDL) particles as well as the total plasma levels of the HDL apolipoproteins were decreased significantly in zinc-deficient rats.

The above-cited observations and the present finding of a marked decrease in the apo-B content of lymph chylomicrons in zinc-deficient rats strongly suggest that the limited availability of apo-B during chylomicron formation may be primarily responsible for the massive accumulation of large sizes of lipid droplets in the intestinal epithelium with resulting impairment of lipid transport. The essential role of apo-B in the formation and release of chylomicrons has been demonstrated by the studies on human subjects with congenital β-lipoprotein deficiency (34,35), which is characterized by the inability to synthesize apo-B. The above-described morphological characteristics of cytoplasmic lipid droplets (5) in association with impaired intestinal transport of lipid in zinc deficiency (1,5) are remarkably similar to those described in human β-lipoprotein deficiency (36) and in rats treated with protein-synthesis inhibitors (18).

However, the morphological aberrations in chylomicrons and impaired lipid transport in zinc deficiency, as observed in the earlier (1,5) and present studies, may not be attributable solely to the lack of apo-B. The present data do not exclude the possibility that such defects also may be due to a general lack of other surface constituents including phospholipid and free cholesterol or the limited contents of surface materials in relation to the hydrophobic core constituents of chylomicrons such as triglyceride and cholesterol ester. In this experiment, chylomicron cholesterol was not partitioned into free and esterified cholesterol. However, the ratio of triglyceride (TG) to PL content previously has been shown to be highly correlated with the size of chylomicrons (37). In this study, the TG/PL ratio of lymph chylomicrons tended to be higher in ZD rats as compared with PF and CT controls (Table 4) but the difference between ZD and controls was not statistically significant ($p > 0.05$). However, the ratio of TG to PL plus protein was significantly higher in ZD rats (23.0 ± 1.9) as compared with 18.8 ± 1.0 in PF and 18.0 ± 1.0 in CT controls. This observation is consistent with the increase in the size of chylomicrons appearing in the lacteal of ZD rats (Fig. 1). This suggests that the formation of large-size chylomicrons in zinc deficiency may be partly due to the diminished availability of both surface lipid and protein during chylomicron assembly within the intestinal epithelium.

In summary, this study provides evidence that zinc deficiency, even at a marginal level, produces a marked decrease in the apo-B content of chylomicrons with an increase in the ratio of the core-to-surface constituents of chylomicrons. Such molecular changes in chylomicrons suggest a defect in the mucosal synthesis of apo-B and surface materials of chylomicrons in zinc deficiency. This may lead to the defective formation and release of chylomicrons from the mucosa, hence the impairment in intestinal absorption of lipids (1,7) and possibly lipid-soluble vitamins, which require chylomicrons as their principal carriers. Some of the clinical manifestations of zinc deficiency may be associated partly with such absorptive defects.

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Effects of Dietary Primrose Oil on Mammary Tumorigenesis Induced by 7,12-Dimethylbenz(a)Anthracene

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**The mammary tumor–promoting effect of a high-fat diet containing 20% evening primrose oil (PO) was compared to that of a 20% corn oil (CO) diet. Mammary tumors were induced in female Sprague–Dawley rats using 10 mg (Study 1) and 5 mg (Study 2) 7,12-dimethylbenz(a)anthracene (DMBA). The 10 mg dose of DMBA gave a total mammary tumor incidence of 47% in rats fed the PO diet and 80% for those fed the CO diet. When only adenocarcinomas were counted, the malignant mammary tumor incidences were 41% in rats fed the PO diet and 73% in rats fed the CO diet. In a second study using 5 mg DMBA to induce mammary tumors, total tumor incidences were 50% for PO-fed rats and 63% for those receiving a CO diet. Again, when only adenocarcinomas were counted, tumor incidences were 27% for PO- and 63% for CO-dieted rats. Analysis of plasma fatty acid profiles indicated that animals fed a 20% PO diet showed significant increases in 18:3 and 20:4 fatty acids and significant decreases in 16:0 and 18:1 compared to animals fed a 20% CO diet. These results indicate that the mammary tumor promoting effect of a diet containing 20% fat can be diminished by substituting PO for CO. Moreover, the promoting effect on mammary cancer by a high-fat diet could be depressed by feeding a source of γ-linolenic acid (GLA).
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The stimulating effect of a high-fat diet on both spontaneous and carcinogen-induced mammary tumorigenesis in rodents now is well-established (1-8). The mechanism(s) by which a high-fat diet causes this stimulation is not fully understood. However, experimental evidence shows that dietary fat rich in polyunsaturated fat (especially *cis*-linoleic acid) influences tumor growth during the promotional phase of mammary carcinogenesis (3,9-11).

The effect of dietary fat may be mediated by a change in the host defense system. One mechanism by which this could happen is by altering synthesis of eicosanoids. Corn oil (CO) and primrose oil (PO) respectively contain 60% or 75% linoleic acid, the precursor of arachidonic acid and ultimately of the dienoic eicosanoids (prostaglandins) and polyenoic eicosanoids (leukotrienes) (12).

In addition to 75% linoleic acid, PO contains 9% of γ-linolenic acid (GLA). GLA is rapidly elongated to dihomo-gamma-linolenic acid (DGLA) the precursor of monoenoic eicosanoids. Ghayur and Horrobin (13) have shown that the growth rate of a transplanted R3230KAC mammary tumor was significantly reduced in female Fisher rats treated daily with different levels of PO. They speculated that prostaglandin E_1 might be able to inhibit mammary tumor growth.

In other studies, Dippenaar et al. (14,15) and Leary et al. (16) have shown that growth in three malignant tumor cell lines (B16 melanoma, human esophageal carcinoma and human hepatoma) was inhibited by GLA-supplementation. They suggested that malignancy may be related to a GLA-deficiency. Karmali et al. (17) provided evidence that PO inhibits the growth of the R3230AC mammary tumor. When pure GLA was given by gavage to tumor-bearing rats, no effect on tumor growth was seen. However, the maximum dose of pure GLA given was below the limit at which tumor inhibition was observed in the rats given GLA in PO (17).

This study was conducted to compare the effects of a diet containing 20% PO to a diet containing 20% CO on the incidence and growth of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumorigenesis as well as on plasma lipid profiles.

MATERIALS AND METHODS

Animals, diets and tumor induction. In two separate studies, 40-day-old virgin female Sprague-Dawley rats (Harlan, Madison, WI) were maintained on Purina Lab Chow (No. 5001) and housed in suspended metal cages in a temperature-regulated (23 ± 0.5 C) and light-controlled (12 hr light and 12 hr dark) room. Food and water were available ad libitum. At 50 days of age, the rats (weighing 179 ± 7.9 g) each were given a single intragastric dose of 5 or 10 mg of DMBA (Sigma Chemical Co., St. Louis, MO) in 0.5 ml CO. In Study 1, the rats were given 10 mg DMBA and divided into two groups of 18 each. In a subsequent study (Study 2), more animals were used to determine if the same or greater effect on tumorigenesis would be noted with a smaller dose of carcinogen. In this study, the rats were given 5 mg DMBA and divided into two groups of 30 each. After DMBA administration, the animals were placed randomly on Absorb Dri litter in plastic cages and housed two per cage for the duration of the experiment. At 21 days (Study 1) and 14 days (Study 2) post-DMBA administration, rats were randomly divided into two groups and fed diets containing either 20% CO or 20% PO. Ten kilogram batches of the 20% fat diets were prepared and cold-pressed into jumbo pellets as needed by ICN Nutritional Biochemical (Cleveland, OH) using the formulation shown in Table 1. In our laboratory, 1 kilogram bags of pellets were placed in Seal-N-Serve bags, flushed with nitrogen, sealed and stored frozen until used. The fatty acid composition of the CO and PO diets are given in Table 2.

Tumor measurements and classification. Rats were weighed and palpated weekly for the presence of mammary tumors, and the size and location of each tumor were noted. Three out of 18 of the rats from Study 1 fed a CO diet and 1 out of 18 of the rats fed a PO diet were found dead before the experiment's termination. No rats in Study 2 died before termination of the experiment.

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Abbreviations: AA, arachidonic acid; CO, corn oil; DGLA, dihomo-gamma-linolenic acid; DMBA, 7,12-dimethylbenz(a)anthracene; GLA, γ-linolenic acid; LA, linoleic acid; PO, evening primrose oil.

TABLE 1

Composition of the Basic Rat Diet

Ingredient	Weight (gm/100 gm)
Fat[a]	20.0
Casein (Vitamin-free)	20.0
Sucrose	25.0
Corn starch	25.0
Alphacel	5.0
AIN Mineral Mix	3.5
AIN Vitamin Mix[b]	1.0
Choline bitartrate	0.2
dl-Methionine	0.3

[a]Primrose oil was given by Efamol Research Inc., Nova Scotia, Canada. ICN purchased corn oil from Seaway Foods, Inc., Cleveland, OH.
[b]Alpha-tocopherol was added with the AIN vitamin mix so that each diet contained a total of 110 IU of vitamin E/kg (DL-α-tocopherol powder, 250 IU/gm) of diet.

TABLE 2

Percent Composition of Fatty Acid in Rat Diets

Fatty acid chain length	% Fatty acid in oil	
	Corn	Primrose
16:0	11.2	6.5
18:0	2.1	1.5
18:1n-9	25.0	7.5
18:2n-6	59.9	75.0
18:3n-6	0.5	9.0
Others	0.1	0.5

At 16 or 13 weeks post-DMBA, surviving rats were killed with CO_2, blood was drawn from the heart for plasma lipid analysis, tumors were excised, counted and weighed, fixed in 10%-buffered formalin, processed for paraffin embedding, sectioned, and stained with hematoxylin and eosin for histological examination. Mammary tumors were identified according to the criteria of Van Zwieten (18). Both benign tumors (fibroadenoma and cystadenoma) and malignant tumors (adenocarcinomas, e.g. tubulopapillary carcinoma) were reported.

Lipid analysis. Total lipid extracts were prepared by extraction with chloroform/methanol (2:1, v/v) after the method of Folch et al. (19). Briefly, a 0.5 ml aliquot of plasma was added to 12 ml of chloroform/methanol, mixed and the extract washed with 2.5 ml of 0.5% sulfuric acid. The aqueous layer was removed by aspiration; aliquots of the organic layer were dried under nitrogen for preparation of fatty acid methyl esters.

Methyl esters of fatty acids were prepared with boron trifluoride following the method of Morrison and Smith (20). A 3.0 ml aliquot of the total lipid extract was dried under nitrogen, and 1.0 ml of boron trifluoride in methanol (14 g/100 ml) was added. The tubes were sealed and heated at 100 C for 60 min. The reaction was stopped by addition of 1.0 ml of distilled-deionized water; the fatty acid methyl esters were extracted with 10 ml of hexane. The hexane extracts were sealed in tubes under nitrogen and stored at −20 C before analysis.

TABLE 3

Effect of 20% Fat Diet on Body Weight in Rats 16 Weeks Post-DMBA

Diet	Number of rats	Dose of DMBA	Final body wt[a] (g)
CO	30	5 mg	282.5 ± 3.4
PO	30	5 mg	286.9 ± 3.2
CO	15	10 mg	278.5 ± 6.2
PO	17	10 mg	267.1 ± 5.6

[a]Values are means ± SEM.

The fatty acid methyl esters were analyzed using a Hewlett-Packard Model 402 gas chromatograph equipped with flame ionization detector and connected to a Spectra-Physics Model 4270 recording integrator. Chromatography was performed on a 6 ft × 2 mm glass column containing 10% SP-2330 on 100/120 chromosorb W AW (Supelco Inc., Bellefonte, PA).

Statistical analysis. Mammary tumor incidence and plasma fatty acids were compared using Student's t-test. The relationship between the type (malignant vs benign) of tumor incidence and the high fat diets were analyzed using chi-square analysis.

RESULTS

The effects of feeding diets containing 20% PO and 20% CO on weight gain in rats treated with DMBA are shown in Table 3. There were no significant differences in weight gain between rats fed the two diets. This was as expected since neither diet was essential fatty acid nor nutrient-deficient.

As expected, the average number of tumors and tumor-burden per tumor-bearing rat (Table 4) in Study 1 (10 mg DMBA) was significantly greater than in Study 2 (5 mg DMBA). The average latency period was significantly longer in Study 1 than in Study 2. However, in Study 2 the 20% fat diets were begin two weeks post-DMBA rather than three weeks post-DMBA as in Study 1.

Table 5 summarizes necropsy data from Studies 1 and 2. In Study 1, in which rats were placed on a diet three weeks post-DMBA (10 mg), eight of 17 rats fed a PO diet developed tumors, whereas 12 of 15 rats fed CO developed tumors. When tumors were classified histopathologically at necropsy (16 wks post-DMBA), 34 malignant tumors were recovered from rats fed CO; only 20 were recovered from rats fed the PO diet. In addition, the total number of tumors in the PO-fed rats was 31, while there were 61 tumors in the CO-fed rats. In the second study (5 mg DMBA), 15 of 30 rats fed the PO diet had tumors, and 19 of 30 rats fed the CO diet had tumors. There were totals of 33 tumors in the PO groups and 36 tumors in the CO groups. However, 15 of 33 tumors were adenocarcinomas (tubulopapillary carcinomas) in the PO group and 36 of 36 tumors were adenocarcinomas in the CO group. Chi-square analysis of the tumor data (Table 5) showed

TABLE 4

Mammary Tumor Incidence in DMBA-Treated Rats Fed Diets Containing 20% Primrose Oil (PO) or 20% Corn Oil (CO)

Diet	Dose of DMBA	Number of surviving rats	Number of rats with tumors	Average number of tumors/tumor bearing rat[a]	Average latency period (wk)[a,b]	Tumor burden/ tumor-bearing rats (g)[a]
PO	5 mg	30	15	2.2 ± 0.2	10.4 ± 0.6	1.20 ± 0.45
CO	5 mg	30	19	1.9 ± 0.2	9.1 ± 0.6	0.95 ± 0.43
PO	10 mg	17	8	3.9 ± 0.8[c]	12.4 ± 0.4[c]	9.18 ± 3.37[c]
CO	10 mg	15	12	5.1 ± 1.1[c]	12.1 ± 0.4[c]	9.82 ± 4.67[c]

[a]Values are mean ± SEM.

[b]Weeks from time of DMBA administration to appearance of first palpable tumor.

[c]Values obtained when 10 mg of DMBA was used to induce mammary tumors were significantly different from values obtained when 5 mg of DMBA was used at p-values ranging from $p < 0.05$ to $p < 0.005$.

TABLE 5

Histopathology of Mammary Tumors in DMBA-Treated Rats Fed Diets Containing 20% Primrose Oil (PO) or Corn Oil (CO)

Diet	Dose of DMBA	Number of surviving rats	Number of rats with tumors	Total number of tumors	Number of rats with benign tumors[a]	Number of benign tumors	Number of rats with malignant tumors[b]	Number of malignant tumors
PO	5 mg	30	15	33	7	18	8	15
CO	5 mg	30	19	36	0	0	19	36
PO	10 mg	17	8	31	5	11	7	20
CO	10 mg	15	12	61	5	27	11	34

[a]Benign tumors = fibroadenoma and cystadenoma.

[b]Malignant tumors = tubulopapillary carcinoma.

TABLE 6

The Effect of Dietary Fat on Plasma Fatty Acid Profiles in Rats Treated with 10 mg DMBA

Diet	Number of plasma samples	% Fatty acid composition[a]						
		16:0	18:0	18:1	18:2	18:3	20:3	20:4
20% Corn oil	15	16.2 ±0.92	14.73 ±1.39	12.53 ±1.11	30.0 ±2.27	0.15 ±0.13	N.D.*	26.67 ±1.58
20% Primrose oil	17	12.69[b] ±0.79	16.0 ±0.52	5.44[b] ±0.47	32.19 ±0.90	2.81[b] ±0.26	N.D.*	30.56[c] ±1.09

[a]Values are mean ± SEM.

[b]Significantly different compared to CO at $p < 0.01$.

[c]Significantly different compared to CO at $p < 0.025$.

*N.D. = not detectable by integration.

that the tumor incidence obtained (malignant vs benign) was dependent on the type of fat being fed. Therefore, feeding PO significantly decreased malignant tumors compared to feeding CO.

Plasma fatty acid profiles at necropsy are shown in Table 6. Linoleate levels in the plasma of rats fed 20% PO and 20% CO were similar; GLA (18:3) and arachidonate (20:4) levels were significantly higher, and oleic acid levels were significantly lower in rats fed PO compared to those fed CO.

DISCUSSION

In two studies using different levels (5 and 10 mg) of DMBA, the total numbers of mammary tumors, malignant tumors and rats with tumors were reduced in rats fed a 20% PO diet compared to rats fed a 20% CO diet. The average number of tumors per tumor-bearing rat and the average tumor-burden per rat were not different significantly in rats fed the two diets. When the two doses of DMBA (5 and 10 mg) were compared, there were

significant differences in these parameters. This result shows that DMBA-induced mammary tumorigenesis was dose-dependent in rats fed both PO and CO diets. However, there was a significant increase in the average tumor latency in Study 1 vs Study 2. It appears that beginning the high fat diet a week earlier in Study 2 shortened the average tumor latency by one week. The plasma fatty acid analyses reflected the fatty acid composition of the oils being fed. Feeding the rats a source of GLA in PO resulted in a plasma pool of GLA 19 times that of feeding CO.

Experiments by Chan et al. (21), Ip et al. (22) and Tinsley et al. (23) have shown that mammary tumorigenesis in rats and mice is positively correlated with the linoleate content of the dietary fat. In this study, the PO diet contained 14% more linoleate than the CO diet, yet tumorigenesis was suppressed. However, PO contains 9% GLA, in addition to 75% linoleic acid (LA). The metabolic pathways for LA, 18:2n-6 are well-established. LA is converted by Δ-6 desaturase (a rate-limiting enzyme) to GLA, 18:3n-6, which is rapidly elongated to DGLA, 20:3n-6 (24). DGLA subsequently is desaturated by Δ-5-desaturase (a rate-limiting enzyme) to arachidonic acid (AA, 20:4n-6), which is converted by fatty acid cyclooxygenase to the dienoic eicosanoids (25). DGLA also is used as a substrate by cyclooxygenase to form the precursor of the monoenoic eicosanoids (12).

It has been shown that an increasing ratio of DGLA/AA may shift eicosanoid synthesis toward monoenoic eicosanoids. This may be beneficial in a number of pathological states by depressing 2-series and/or by enhancing the production of the 1-series eicosanoids (26). PGE_2 has been shown to suppress the immune response and is present in high levels in mammary tumors (12). Therefore, manipulation of the DGLA/AA balance may provide a selective regulation of eicosanoid synthesis, which could be helpful in cancer treatment and/or prevention. Further studies are being conducted to examine the mechanism(s) responsible for the reduction of DMBA-induced malignant mammary tumors in rats fed PO.

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METHODS

Extraction and Quantitation of Total Cholesterol, Dolichol and Dolichyl Phosphate from Mammalian Liver

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A procedure is described for the determination of total cholesterol, dolichol and dolichyl phosphate (Dol-P) in mammalian liver. It is based on extraction of these compounds into diethyl ether after alkaline saponification of the tissue. Extractability is affected by the length of saponification and concentration of potassium hydroxide (KOH) in the saponification mixture. After extraction, total cholesterol and dolichol are quantitated directly by reverse-phase high pressure liquid chromatography (HPLC) on C_{18}. Dol-P requires further purification before quantitation by HPLC, this is accomplished by chromatography on silicic acid. These methods gave recoveries of over 90% for cholesterol and dolichol and about 60% for Dol-P, using [4-^{14}C]cholesterol, a polyprenol containing 15 isoprene units, and [1-^{14}C]Dol-P as recovery standards. Concentrations of total cholesterol, dolichol and Dol-P in livers from one month-old-CBA mice were found to be 5.7 ± 0.7 mg/g, 66.3 ± 1.2 μg/g and 3.7 ± 0.3 μg/g, respectively.
Lipids 22, 1045-1048 (1987).

Dolichyl phosphate (Dol-P) is an obligatory intermediate in the dolichol cycle by which many secretory and membrane-bound N-linked glycoproteins are produced (1). Its concentration can be an important regulatory factor in the formation of these glycoproteins (2-5).

Dolichol and cholesterol readily can be extracted from tissues and quantitated but determination of total Dol-P has proven more difficult. Dol-P was first measured by Dallner et al. (6) using enhancement of Dol-P-mannose formation by a Dol-P-dependent mannosyl transferase following addition of a purified Dol-P-containing fraction. Carson and Lennarz (7) subsequently separated Dol-P from dolichol, enzymatically dephosphorylated it and quantitated it by high performance liquid chromatography (HPLC). Two methods for the direct quantitation of Dol-P by HPLC have been developed. Chaudhary et al. (8) used a reverse-phase C_{18} column, and Keller et al. (9) used a straight-phase silica column.

The problems encountered in the quantitation of Dol-P have been attributed to the extraction and purification before analysis by HPLC (10). Sequential extraction with chloroform/methanol (2:1, v/v) and chloroform/methanol/water (10:10:3, v/v/v) may give incomplete extraction of total Dol-P from rat liver, and conversion of Dol-PP-oligosaccharide to free Dol-P by acid hydrolysis may not always go to completion (9,11). A recent report (9) indicates that total cholesterol, total dolichol and total Dol-P can be extracted from a potassium hydroxide (KOH) saponification mixture into diethyl ether (ether). Dol-PP, and presumably its glycosylated forms, are converted to free Dol-P under these conditions. These workers also reported that the levels of Dol-P in rat tissues are comparable to or higher than the levels of dolichol. Their values for Dol-P are several times higher than those based on other methods of extraction (5,8,12, 13). Furthermore, levels of dolichol reported by Keller et al. (9) are often lower than those observed previously.

Studies in our laboratory indicated that Dol-P was extracted inefficiently from saponification mixtures (14,15). Therefore, we further investigated the extraction of cholesterol, dolichol and Dol-P following saponification of mammalian tissue. We now describe a method for isolation and quantification of these compounds that eliminates the need for separation of cholesterol from dolichol before HPLC analysis and that yields good recoveries of all three compounds.

EXPERIMENTAL

Materials. Mice (one-month-old National Institute of Aging CBA/Ca males) were obtained from Charles River Breeding Laboratories (Wilmington, MA). Human liver was obtained at autopsy. [1-^{14}C]Dolichol (50 mCi/mmol), consisting of a series of homologues containing 15 to 24 isoprene units (18 and 19 predominating), was a gift from the Kuraray Co. (Okayama, Japan). [1-^{14}C]Dolichyl phosphate was prepared according to the method of Danilov and Chojnacki (16) and purified by chromatography on silicic acid (Bio-Rad Laboratories, Richmond, CA). [4-^{14}C]-Cholesterol (57.5 mCi/mmol) of >99% radiochemical purity was purchased from New England Nuclear (Boston, MA). Cholesterol (>99% pure) was obtained from Calbiochem (La Jolla, CA). Polyprenol-15 (α-unsaturated) was obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of at least reagent grade.

Saponification and extraction. Two methods of saponification and extraction were compared. Method 1, described by Keller et al. (9) for assay of free-and chemically bound Dol-P uses direct saponfication of tissue followed by ether extraction and quantitation by HPLC. The tissue (1 g) was saponified in a boiling water bath for 60 min in a mixture of 1 ml of 60% KOH (w/v) and 2 ml of methanol containing 0.25% pyrogallate. The mixture then was extracted with ether as described by Keller and Adair (17).

Method 2 is a modification of that of Burgos et al. (18), routinely used in our laboratory (14,15). In this case, the 1g tissue samples were saponified for one, two or sixteen hr in a boiling mixture containing 0.3 g KOH, 0.2 ml water and 0.2 ml ethanol followed by ether extraction. In this modified method, pyrogallol was omitted because it is reported to be incompatible with alkaline solutions (19). [1-^{14}C]Dolichol or [1-^{14}C]Dol-P were added as tracers in each case. The final concentrations of KOH were about 15% in Method 1 and 25% in Method 2, assuming liver tissue was 80% water.

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Abbreviations: Dol-P, dolichyl phosphate; HPLC, high pressure liquid chromatography; KOH, potassium hydroxide.

On the basis of experience with these two methods, Method 3 was developed as follows: 1 g aliquot of liver was saponified for 16 hr (overnight) in a boiling mixture of 2 ml of 90% KOH (w/v) and 1 ml methanol. The mixture was allowed to cool to room temperature and was extracted three times with equal volumes of ether. The ether extracts were pooled and washed with 4.1 ml of water. The saponification mixture then was diluted to 15% KOH with the water wash plus 4.1 ml of methanol and reextracted three times with 10 ml of ether. The second set of extracts were pooled and washed with 10 ml of 5% acetic acid. Both pooled extracts were dried under N_2. The first ether extract, containing the cholesterol and dolichol, was taken up in chloroform/methanol (2:1, v/v) for analysis by HPLC. The second extract, containing the Dol-P, was resuspended in chloroform and loaded on a 1 g column of silicic acid. The column was washed with 5 ml of chloroform, and the Dol-P was eluted with 10% methanol in chloroform. The eluate was dried under N_2 and taken up in chloroform/methanol (2:1, v/v). Aliquots were taken for determination of radioactivity and analysis by HPLC.

Quantitation by HPLC. Cholesterol, dolichol and Dol-P were analyzed on a Hewlett-Packard 1084-B HPLC using a reverse-phase C_{18} column (Hewlett-Packard #799150d-174) equipped with a variable wavelength detector set to read absorbance at 210 nm with 430 nm as a reference wavelength (8). The mobile phase consisted of a methanol/isopropanol gradient with increasing proportions of isopropanol delivered at 2 ml/min. The gradient used for the cholesterol/dolichol fraction consisted of seven minutes at 1% isopropanol, increasing to 25% over the next 10 min, holding at 25% for three min and increasing to 70% over the next 10 min. For Dol-P, the gradient was increased from 1 to 50% isopropanol over the first six min and then to 70% over the next 14 min of the run. In this case, the isopropanol contained 10 mM phosphoric acid. Quantitation was achieved by summing the areas of all homologous peaks and comparing to human liver dolichol standards. Cholesterol was quantitated by comparison to the cholesterol standard. Radioactivity in the eluate was counted on an LKB 1219 Rackbeta liquid scintillation counter.

RESULTS

Figure 1a shows that when Method 1 is used, dolichol and Dol-P are extracted efficiently from the saponification mixture after a 1 hr saponification. Recoveries of both labeled compounds in the ether extracts were ca. 90% compared to 30% for dolichol and less than 10% for Dol-P in Method 2 (Fig. 1b). This difference was not due to the presence or absence of pyrogallate as the extraction of dolichol and Dol-P was only slightly less efficient when the pyrogallate was omitted in Method 1 (data not shown).

After a 2 hr saponification, about 85% of both dolichol and Dol-P was extracted by the ether in Method 1, with or without pyrogallate. Using Method 2, the longer saponification greatly increased the extraction of dolichol but had little effect on the extraction of Dol-P (Fig. 1c, 1d). Similar results were obtained following a 16-hr-saponification.

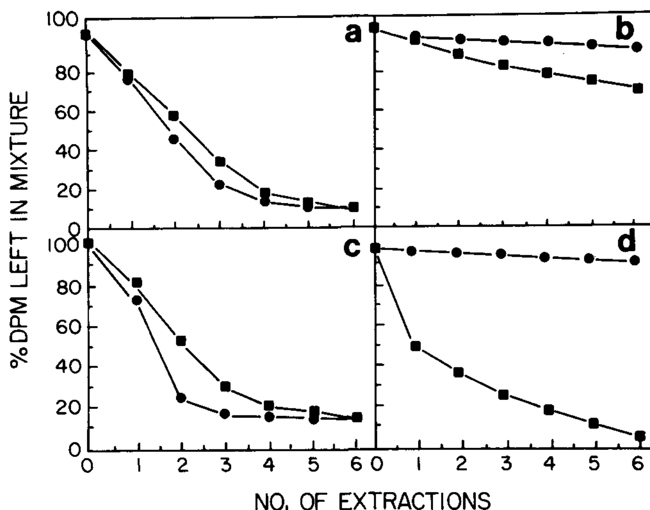

FIG. 1. Percentage of total DPM of [1-^{14}C] dolichol (■) and Dol-P (●) left in the saponification mixture after extraction with ether. Each point represents the average of three observations. (a) One-hr saponification, Method 1. (b) One-hr saponification, Method 2. (c) Two-hr saponification, Method 1. (d) Two-hr saponification, Method 2.

The difference in the extractability of Dol-P in the two methods evidently is related to the concentration of KOH in the saponification mixture. As illustrated in Figure 2, the extraction of Dol-P was improved by diluting with 50% ethanol the KOH in the saponification mixtures from 25% to 15%. Further dilution did not increase the amount of Dol-P extracted. If methanol was substituted for ethanol in Method 2 and the mixture diluted to 15% KOH after saponification, Dol-P was extracted as efficiently as in Method 1.

Saponification of tissue with Method 3's strong KOH solution for a 16 hr period ensured that dolichyl fatty acyl esters in the tissue were hydrolyzed to dolichol. Dolichyl esters were hydrolyzed incompletely after a two-hr saponification of liver tissue (data not shown) or a 17-hr saponification of soybean embryos (Rip and Carroll, unpublished data). The cholesterol and dolichol could be extracted from this saponification mixture with ether, and the extract was suitable for HPLC analysis without further purification (Fig. 3a) even though about 5% of the Dol-P remained in the fraction. This does not interfere with the quantitation of the cholesterol or dolichol as small amounts of Dol-P run in the solvent front when phosphoric acid is omitted from the eluting solvent.

Subsequent dilution of the saponification mixture to 15% KOH made it possible to extract the Dol-P with ether but further purification was required before HPLC analysis. This was necessary to remove residual dolichol (ca. 2% of the total dolichol in the tissue) in the ether extract. The contaminating dolichol amounted to one-third to one-half of the total Dol-P and interfered with quantitation of the Dol-P.

Attempts to purify the Dol-P by back extraction from ether into water did not separate it from the dolichol as cleanly as in experiments on soybean embryos (10). Therefore, the dolichol was removed by silicic acid chromatography, and this gave a Dol-P fraction that could be analyzed by HPLC (Fig. 3b). However, approximately 20%

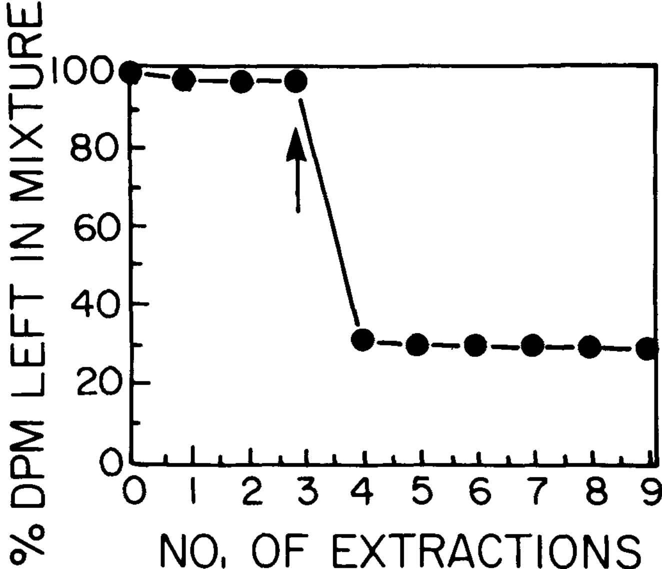

FIG. 2. Percentage of total DPM of [1-^{14}C]Dol-P left in the saponification mixture after extraction with ether. Saponification was done over two hr using Method 2. Each point represents the average of three observations. The arrow indicates the point where the saponification mixture was diluted with 50% ethanol from 25% KOH to 15% KOH.

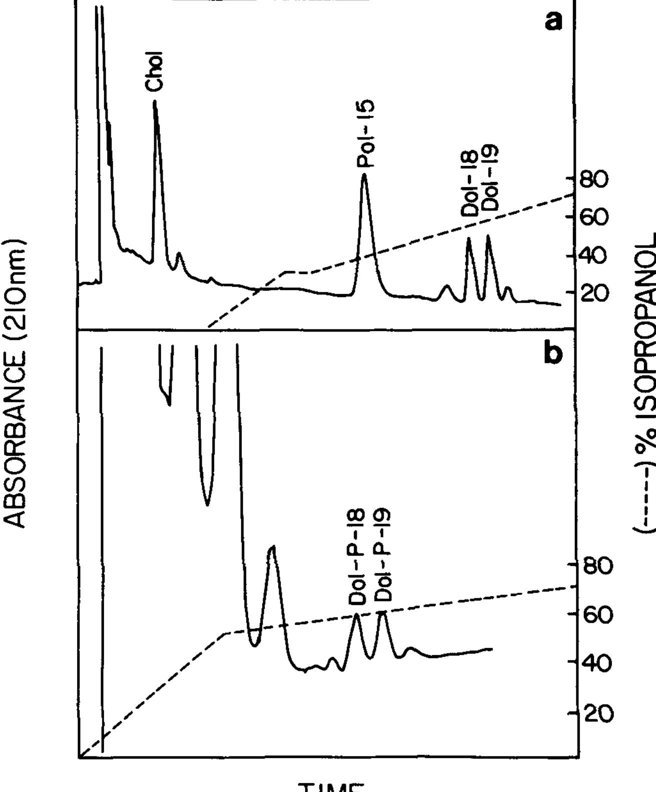

FIG. 3. C_{18} reverse-phase HPLC chromatograms using methanol/isopropanol gradients. (a) CBA/Ca mouse liver extract, cholesterol/dolichol fraction. Absorbance units/full scale deflection decrease by eight-fold between the cholesterol peak and the polyprenol peak. (b) CBA/Ca mouse liver extract, Dol-P fraction. The isopropanol in the eluting solvent contains 10 mM phosphoric acid.

of the tracer Dol-P extracted from the saponification mixture bound tightly to the column and could not be recovered in the eluate. The reason for this is unclear but it is possible that some of the Dol-P is oxidized by the treatment.

The recoveries of cholesterol, dolichol and Dol-P were 92 ± 1%, 94 ± 4% and 61 ± 1% (SEM, n = 4), respectively. Analysis of mouse liver using Method 3 gave values of 5700 ± 700 μg/g of cholesterol, 66.3 ± 1.2 μg/g of dolichol and 3.7 ± 0.3 μg/g of Dol-P on a wet weight basis.

DISCUSSION

Our values for the concentrations of dolichol and Dol-P in mouse liver are similar to those reported for liver of male C57BL/6J mice one month after weaning (20). The concentration of dolichol also is similar to that reported for liver of 12-month-old C57 B1/6 mice (21). Dol-P accounted for about 5% of the sum of the forms of dolichol in the liver, which is somewhat less than the values of 18% and 6.7% reported for mouse liver at weaning and one month after weaning, respectively (20), or values of 7%, 14% and 16% in rat liver as determined by other methods (5,22,23). Our results differ considerably from those of dolichol, Dol-P (17.1 and 14.7 μg/g, respectively) and the percentage present as Dol-P (46%) reported for rat liver by Keller et al. (9). The reason for this discrepancy is not clear but it seems unlikely to be due to species differences.

Keller et al. (9) attributed the high levels of Dol-P found in their experiments to a more efficient extraction of Dol-P from the saponification mixture. Our studies confirm that Dol-P is extracted efficiently from saponification mixtures when the concentration of KOH is appropriate, but the concentration of Dol-P in the liver is still much lower than that reported by Keller et al. (9). It is possible that the difference is related to the use of different methods of analysis by HPLC, although this does not seem very likely.

There is considerable interest in the metabolism and function of dolichol and its derivatives. Therefore, it is desirable to have a simple and reliable method for extraction and quantitation of dolichol and Dol-P. Our studies have indicated that prolonged saponification may be required for hydrolysis of dolichyl acyl esters (unpublished data). The extraction of Dol-P from the saponification mixture by ether is dependent on the concentration of KOH; this can be used to isolate the dolichol and Dol-P separately for quantitation by HPLC. The method described gives excellent recoveries of cholesterol and dolichol in a form that can be analyzed directly by HPLC. The recovery of Dol-P is lower but still much better than that obtained with the earlier method used in our laboratory (8).

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Analysis of Disteryl Ethers

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Disteryl ethers isolated from a vegetable oil and a table margarine were analyzed by capillary gas chromatography. The composition of disteryl ethers in such products reveals whether vegetable oils are mixed with animal fats and if these materials have been subjected to industrial bleaching.
Lipids 22, 1049-1052 (1987).

Disteryl ethers are minor but ever-present constituents of industrially refined fats and oils (1-3). They are artifacts generated from plant and animal sterols during the bleaching process (4). Moreover, disteryl ethers are formed by various acid-catalyzed reactions (1). Methods for the thin layer chromatography (TLC) separation of mixtures of disteryl ethers from the unsaponifiable fractions of fats and oils are described in the literature (4-6). However, little is known about methods for the analysis of the individual components of these mixtures.

$R_1 = R_2$:uniform disteryl ethers
R_1, R_2 = H:dicholesteryl ether
$R_1, R_2 = CH_3$:dicampesteryl ether
$R_1, R_2 = C_2H_5$:disitosteryl ether

$R_1 \neq R_2$:mixed disteryl ethers
R_1 = H; $R_2 = CH_3$:cholesterylcampesteryl ether
R_1 = H; $R_2 = C_2H_5$:cholesterylsitosteryl ether
$R_1 = CH_3$; $R_2 = C_2H_5$:campesterylsitosteryl ether

Structure of various disteryl ethers.

This study describes the separation of various individual disteryl ethers by capillary gas liquid chromatography (GLC). Methods for the analysis of these compounds in refined fats and oils may be of interest with regard to food composition and adulteration. In addition, the method described here may be useful for the determination of disteryl ethers in food and feedstuff as well as of their distribution in organs of man and animal after ingestion.

MATERIALS AND METHODS

Chemicals. Cholesterol, sitosterol and stigmasterol were purchased from E. Merck (Darmstadt, FRG). Crude sitosterol, containing about 40% campesterol, was obtained from Sigma Chemie (Deisenhofen, FRG). A mixture of plant sterols containing brassicasterol, campesterol, stigmasterol and sitosterol (plant sterol mix) was a product of Supelco (Sulzbach, FRG).

Preparation of disteryl ethers. Dicholesteryl ether (melting point [mp] 195-198 C), disitosteryl ether (mp 188-193 C), distigmasteryl ether (mp 207-210 C), as well as a mixture of disteryl ethers derived from cholesterol and sitosterol (1:1, mol/mol), a mixture of disteryl ethers derived from the plant sterol mix, a mixture of disteryl ethers derived from the sterol fraction that was isolated from a vegetable oil, and a mixture of disteryl ethers derived from the sterol fraction that was isolated from a table margarine were prepared by heating (200 C, 15 min) of sterols with anhydrous $CuSO_4$ (7). After cooling, the reaction mixtures were extracted three times with hexane. The solvent was removed, and the resulting mixture of reaction products was treated with ethanol to remove dehydrosterols. The resulting brown residue was purified by repeated crystallization from hexane/acetone (4:1, v/v). The purity of the disteryl ethers (R_f 0.45) was checked by TLC on Silica Gel H (E. Merck) with hexane/diethyl ether (95:5, v/v) (4). Chromatograms were visualized by spraying the plates with chromic-sulfuric acid and charring. Mp (uncorrected) and infrared (IR) spectra of disteryl ethers agreed well with the data given in the literature (4,7).

Isolation of sterols and disteryl ethers from oil and margarine. A commercially available vegetable oil and a table margarine containing a mixture of vegetable fats and animal fats as well as genuine olive oil, 30 g each, were subjected to alkaline hydrolysis according to an established procedure (8). The unsaponifiable fraction was isolated by repeated extraction of the reaction mixture with diethyl ether.

The sterols were separated from the unsaponifiable fraction by precipitation with digitonin (9). The precipitate was isolated, dissolved in dimethyl sulfoxide and extracted three times with diethyl ether. The sterol extract was washed with water and dried; the fraction of sterols was further purified by TLC on layers of silica gel using hexane/diethyl ether (3:2, v/v). The resulting sterol mixture was used for GLC and for preparation of disteryl ethers as described above.

The constituents of the remaining unsaponifiable fraction were separated by repeated preparative TLC on Silica Gel H layers (0.5 mm) with hexane/diethyl ether (95:5, v/v). The fraction of disteryl ethers (R_f 0.45) was identified by co-chromatography with a standard and isolated by extracting the silica gel with water-saturated diethyl ether. The resulting mixture of nearly pure disteryl ethers was used for capillary GLC.

Analyses by capillary GLC. Capillary GLC of sterols and disteryl ethers was carried out in a Varian 3700 instrument (Darmstadt, FRG) equipped with a flame ionization detector. Peaks were identified by retention times using synthetic reference compounds. Peak areas and retention times were determined using a Merck-Hitachi D-2000 integrator (Darmstadt, FRG).

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Abbreviations: TLC, thin layer chromatography; GLC, gas liquid chromatography; BSA, *N,O*-bis(trimethylsilyl)acetamide; TMS, trimethylsilyl; TMCS, trimethylchlorosilane.

TMS ethers of sterols were prepared by reacting the sterols with trimethylchlorosilane (TMCS) and *N,O*-bis(trimethylsilyl)acetamide (BSA) in pyridine (1:5:10, v/v). They were analyzed on a fused silica column, 30 m × 0.325 mm inside diameter, coated with 0.1 μm SE-54 ("DB-5"; ICT, Frankfurt/M., FRG) at a temperature of 250 C, with injector and detector each set at 270 C. Hydrogen (0.5 bar at column inlet; split 1:10) was used as carrier gas and nitrogen (30 ml/min) as purge gas. The reaction mixtures of trimethylsilyl (TMS) ethers (1.5-2.0 μl; 0.1%) were injected without purification.

Disteryl ethers were separated on a glass capillary, 20 m × 0.27 mm id, coated with 0.05 μm SE-30 from Serva (Heidelberg, FRG) at a temperature of 320 C; the injector and detector each were set at 350 C. Hydrogen (1.5 bar at column inlet; split 1:10) was used as carrier gas and nitrogen (30 ml/min) as purge gas. Solutions (1.5-2.5 μl; 0.05%) of disteryl ethers in cyclohexane were injected.

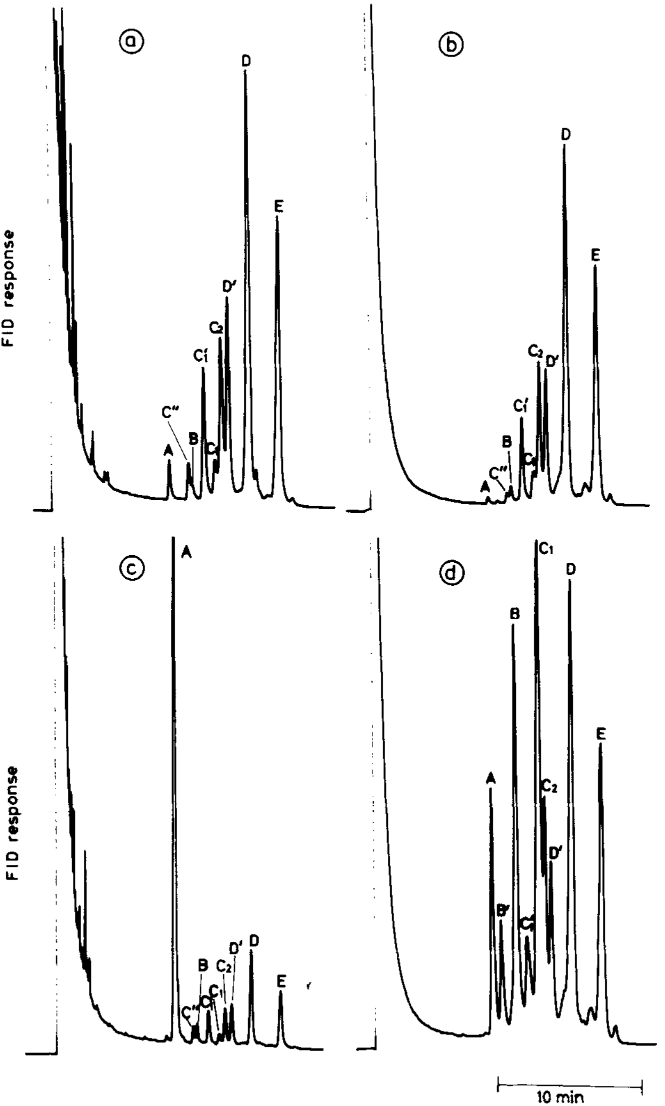

FIG. 1. Capillary GLC of various mixtures of disteryl ethers. (a) Mixture of disteryl ethers isolated from a vegetable oil. (b) Mixture of disteryl ethers that had been synthesized from the sterol fraction isolated from the vegetable oil. (c) Mixture of disteryl ethers isolated from a table margarine. (d) Mixture of disteryl ethers that had been synthesized from the sterol fraction isolated from the table margarine. The disteryl ethers are designated as given in Figure 2. Experimental conditions for GLC are described in *Materials and Methods.* Peaks tentatively are identified from their retention times.

RESULTS AND DISCUSSION

Commercial plant and animal fats and oils pass through various refining steps including deacidification, bleaching and deodorization. Disteryl ethers exclusively are formed during the bleaching process (2,4,5). The formation of these artifactual condensation products is dependent not only on the concentration of sterols in the starting materials and on time, temperature and vacuum during the bleaching process; it also depends on the extent of activation of bleaching earth and its acidity (4,5).

Methods for the analysis of individual disteryl ethers have not been described until now. The separation of dicholesteryl ether from other cholesterol derivatives on a packed column at 240 C has been reported elsewhere (6). However, partial decomposition of disteryl ethers on packed columns at high temperatures was observed by the authors.

In this study, no decomposition of disteryl ethers was detected when capillary GLC was used for the analysis. Figure 1 shows that the most abundant uniform disteryl ethers in refined fats and oils, such as dicholesteryl ether, disitosteryl ether, dicampesteryl ether, distigmasteryl ether, dibrassicasteryl ether and a large number of the corresponding mixed disteryl ethers such as cholesterylsitosteryl ether, campesterylsitosteryl ether and others were resolved sufficiently by capillary GLC (Fig. 2) under the conditions given in *Materials and Methods.*

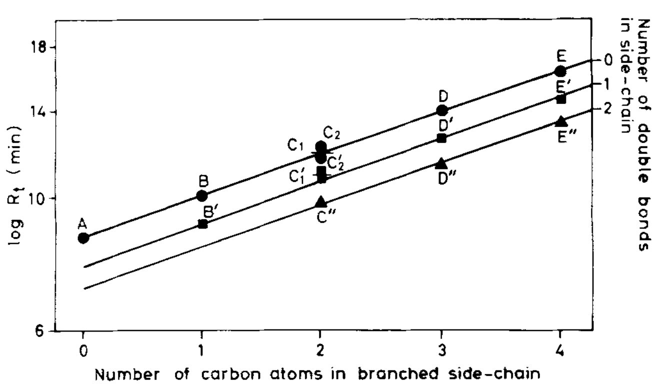

FIG. 2. Retention times, R_t, of various disteryl ethers that had been synthesized from different sterols and sterol mixtures, as a function of carbon atoms and double bonds in the side-chain of steryl moieties. A, Dicholesteryl ether (0:0, number of C-atoms in branched side-chain:number of double bonds in side-chain); B, cholesterylcampesteryl ether (1:0); C_1, cholesterylsitosteryl ether (2:0); C_2, dicampesteryl ether (2:0); D, campesterylsitosteryl ether (3:0); E, disitosteryl ether (4:0); B′, cholesterylbrassicasteryl ether (1:1); C_1', campesterylbrassicasteryl ether (2:1); C_2', cholesterylstigmasteryl ether (2:1); D′, sitosterylbrassicasteryl ether (3:1); E′, sitosterylstigmasteryl ether (4:1); C″, dibrassicasteryl ether (2:2); D″, brassicasterylstigmasteryl ether (3:2); E″, distigmasteryl ether (4:2). Procedures for preparation and isolation of disteryl ethers and experimental conditions of GLC are described in *Materials and Methods.*

It is evident from Figure 2 that retention times of the disteryl ethers increase with the number of C-atoms and decrease with the number of double bonds in the side-chain of the steryl moieties. One might expect that disteryl ethers having a similar arrangement of C-atoms and double bonds in the side-chain, e.g. dicampesteryl ether (2:0) and cholesterylsitosteryl ether (2:0) as well as campesterylbrassicasteryl ether (2:1) and cholesterylstigmasteryl ether (2:1), should show similar retention times; yet, the separation even of such critical disteryl ethers is sufficient under the conditions described (Figs. 1 and 2). In contrast, distanyl ethers and mixed disteryl ethers containing saturated and Δ^5-unsaturated steryl moieties are not separated.

The analysis of disteryl ethers in fats and oils may be of interest with regard to food composition and adulteration. The occurrence of these sterol derivatives proves that refined fats and oils had passed through a bleaching process. Moreover, the analysis of the different disteryl ethers that are formed during commercial processing gives information on whether plant and animal fats and oils have been bleached individually or together. In the former case, the disteryl ether fraction of plant fats would consist of a mixture of disteryl ethers containing only a trace of cholesteryl moieties and that of animal origin would consist almost exclusively of dicholesteryl ether, whereas in the latter case mixed disteryl ethers containing large amounts of cholesteryl moieties would be the main components (Fig. 1).

Analysis of the sterol fraction of the vegetable oil examined here indicates that it may have been derived from rapeseed; however, the table margarine investigated was found to contain a mixture of plant sterols typical for rapeseed oil together with large amounts of cholesterol derived from animal fat (Table 1).

These findings are supported by the composition of disteryl ethers isolated from both the vegetable oil and the table margarine (Table 2). It is obvious that mixed disteryl ethers present in the oil are derived almost exclusively from sterols typical for rapeseed oil (Table 2; Fig. 1a); small amounts of dicholesteryl ether may be formed from traces of cholesterol that have been detected in various vegetable oils (Table 1) (2). The closely resembling pattern of both the disteryl ethers extracted from the vegetable oil (Fig. 1a) and the disteryl ethers synthesized from the sterol fraction of the oil (Fig. 1b) strongly suggests that the vegetable oil does not contain animal fat. However, the table margarine was found to contain large proportions of dicholesteryl ether together with mixed disteryl ethers, the latter having a pattern typical for the disteryl ethers of a refined rapeseed oil (Table 2; Fig. 1a and c). These data clearly indicate that the margarine contained a mixture of fats from animal and plant origin. In contrast, disteryl ethers were not detected in genuine olive oil.

It is commercial practice to blend previously refined fats and oils for the production of margarine. As expected, the results given in Table 1 and Figure 1 show that the constituent vegetable and animal fats of the table margarine must have passed separately through a bleaching process because only traces of mixed disteryl ethers containing cholesteryl moieties were detected in the margarine (Table 2; Fig. 1c). Yet, high proportions of mixed

TABLE 1

Composition of Sterols Isolated from a Commercial Vegetable Oil and from a Table Margarine

Sterols	Vegetable oil[a] (%)	Table margarine[b] (%)
Cholesterol	0.6	27.9
Brassicasterol	9.4	6.1
Campesterol	32.9	25.2
Sitosterol	49.2	34.6
Others[c]	7.9	6.2

[a]Containing 1.9 mg sterols/g oil.

[b]Containing 2.8 mg sterols/g margarine.

[c]Including small amounts of stanols.

TABLE 2

Composition of Constituent Disteryl Ethers Isolated from a Commercial Vegetable Oil and from a Table Margarine

Disteryl ethers	Vegetable oil[a]		Table margarine[a]	
	Found (%)	(Calcd.)[b] (%)	Found (%)	(Calcd.)[b] (%)
Dicholesteryl ether	3	(<1)	—[c]	—[c]
Dibrassicasteryl ether	3	(1)	5	(1)
Dicampesteryl ether	11	(11)	10	(12)
Disitosteryl ether	21	(24)	23	(23)
Campesterylsitosteryl ether	29	(33)	31	(34)
Sitosterylbrassicasteryl ether	13	(9)	12	(8)
Campesterylbrassicasteryl ether	9	(6)	11	(6)
Others[d]	11	(16)	8	(16)

[a]Containing about 1 μg disteryl ethers/g, each.

[b]Calculated for the formation of disteryl ethers from the sterol mixture of the composition given in Table 1.

[c]The disteryl ether fraction of the margarine contains 67% dicholesteryl ether, which was calculated as "100% from animal origin."

[d]Including small proportions of cholesterylcampesteryl ether, cholesterylbrassicasteryl ether, cholesterylsitosteryl ether and traces of disteryl ethers containing stanyl moieties.

disteryl ethers having cholesteryl moieties would be expected if animal and plant fats and oils would have been bleached together. Theoretically, such a mixture of disteryl ethers derived from a sterol fraction (the composition given in Table 1 for the table margarine) should contain as the main components having cholesteryl moieties around 19% cholesterylsitosteryl ether, 13% cholesterylcampesteryl ether and 3% cholesterylbrassicasteryl ether (cf. equation below).

We have simulated such a refining process, in which vegetable and animal fats were bleached together, by preparing a blend of disteryl ethers from the sterol fraction isolated from the table margarine. Analysis by capillary GLC of these mixed disteryl ethers containing cholesteryl moieties showed as the main components cholesterylsitosteryl ether (20%), cholesterylcampesteryl ether (15%) and cholesterylbrassicasteryl ether (4%) (Fig. 1d). These values agree with the calculated data.

For a mixture of four components, a, b, c and d, in which the molecules of each compound are able to react with molecules of the same compound or molecules of each other compound to yield a bimolecular adduct, a theoretical distribution of reaction products has to be expected according to the following equation:

$$(a+b+c+d)^2 = a^2+b^2+c^2+d^2+2(ab+ac+ad+bc+bd+cd).$$

Deviation of our results from the calculated values (Table 2) may be due to further alteration of the sterol composition by other refining steps and differences in the rate of formation of individual disteryl ethers. Thus, we found that under the conditions described in *Materials and Methods* the synthesis of dicholesteryl ether is slightly favored as compared to the synthesis of disitosteryl ether (data not shown). Analysis of disteryl ethers also may be helpful for the detection of part-processed or back-blended vegetable oils that are declared genuine.

Disteryl ethers are formed in many chemical syntheses in which sterols are subjected to acid (1), e.g. transesterification of steryl esters (6) and glycosidation of sterols (10); they also are generated by thermal decomposition of cholesterol 3-sulfate salts (11,12). Obviously, the method described for the analysis of disteryl ethers in fats and oils also may be helpful for the characterization of these compounds if they are formed in various chemical reactions.

Some earlier work has been carried out to study possible biological effects of disteryl ethers, such as cytotoxicity and carcinogenicity (1,13,14). However, little is known on the distribution and metabolism of disteryl ethers in man or animals. The method described for the analysis of these substances may be helpful in the investigation of disteryl ethers and their metabolism in human and animal tissues.

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COMMUNICATIONS

Effects of Eicosatetraynoic Acid on Membrane Lipids of Trout Liver and Intestine

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After two months feeding either an (n-3) or an (n-6) fatty acid-rich diet, two groups of trout were switched to the (n-6) or the (n-3) fatty acid-rich diet, respectively. Half of each group was treated with 0.03% 5,8,11,14-eicosatetraynoic acid (ETYA) in the diet. Liver and intestinal brush border membrane lipids were analyzed. No effect was observed on their cholesterol content. ETYA induced an accumulation of 18:2(n-6), and it did not affect the 20:4(n-6) content but decreased the 22:5(n-6) content. ETYA induced an increase of 18:3(n-3) content in the brush border membrane and a decrease of the 22:6(n-3) content in the liver. Those results suggest that ETYA blocks mainly the Δ6-desaturase, which should have two different sites in the liver and one in the intestine.
Lipids 22, **1053-1056 (1987).**

Eicosa-5,8,11,14-tetraynoic acid (ETYA, Ro 3-1428) is the acetylenic analog of arachidonic acid (1). It first was reported to block cholesterol synthesis between acetate and mevalonate and to exert a suppressive effect on sebaceous gland secretion (2). Later, ETYA also was shown to inhibit arachidonic acid metabolism at the level of cyclooxygenase and lipoxygenase (3). Furthermore, it has been shown to compete with arachidonic acid for esterification enzymes (4). ETYA also has been demonstrated to block the conversion of linoleic acid to arachidonic acid and that of arachidonic acid to more unsaturated (n-6) fatty acids (5).

To analyze in more detail this last effect and the possibility of an influence of ETYA on (n-3) fatty acid metabolism, we investigated the effect of dietary ETYA on the fatty acid composition of phospholipids in the liver and intestinal brush border membranes of rainbow trout. These membranes were selected as easily purified plasma membranes (6) considering their physiological importance and their ability to be modified by dietary treatments (7).

MATERIALS AND METHODS

Fish and membrane preparation. Rainbow trout (250–300 g) were stocked in outdoor tanks that were provided with well-aerated water (12–13 C). Diets were prepared according to Castell et al. (8) and contained 8% linseed oil or grapeseed oil for the (n-3) or (n-6) fatty acid-rich diet, respectively. They were given once daily to the fish (1% body weight) (see Table 1 for the diet's fatty acid composition). The delivered food amount was completely ingested by the fish. Group I was fed the (n-3) fatty acid-rich diet for two months and then switched to the (n-6) fatty acid-rich diet with or without 0.03% ETYA. Group II first was fed the (n-6) fatty acid-rich diet and switched to the (n-3) fatty acid-rich diet with or without 0.03% ETYA. Fish were killed 10 or 30 days after the dietary change. Their livers were quickly removed and frozen. Brush border membranes were purified from scraped mucosa of the middle intestine (6). Membranes of similar purity were selected according to the alkaline phosphatase enrichment (13- to 18-fold).

TABLE 1

Fatty Acid Composition of the Experimental Diets (mol %)

Fatty acid	(n-3) Fatty acid-rich diet	(n-6) Fatty acid-rich diet
16:0	12.9	8.6
18:0	3.2	3.8
18:1(n-9)	16.1	17.8
18:1(n-7)	0.5	0.6
18:2(n-6)	18.0	69.2
18:3(n-6)	0.7	—
18:3(n-3)	48.5	—

Membrane composition studies. Total lipids were extracted from the preparations according to Folch's procedure (9). Liver phospholipids were separated from the crude lipid extract as described previously (6). Individual phospholipids from intestinal membranes were separated by thin layer chromatography according to the method of Fine and Sprecher (10). The data presented here concern only phosphatidylethanolamine (PE) and phosphatidylcholine (PC) because those two phospholipids represent about 60% of the membrane phospholipids and are the most sensitive to dietary alterations (11). Fatty acid methyl esters (12) were analyzed using a Perkin-Elmer Sigma 1 gas chromatograph equipped with a bonded fused silica open tubular column (0.32 mm id × 50 m; Superox, Alltech France, Paris, France). Peak areas were determined by the in-line Perkin-Elmer Sigma 10 chart integrator. Results are given as molar percentages of the total fatty acids. Cholesterol (13) and phospholipid phosphorus (14) were determined on total lipid extracts.

RESULTS

Whatever the diet and treatment, the relative content of cholesterol, phospholipid and proteins was not affected by ETYA (0.6 mg total lipids and 0.4 mg total lipids/mg protein in brush border membrane and liver, respectively). A significant increase in both 18:2(n-6) and 20:3(n-6)

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; EYTA, 5,8,11,14-eicosatetraynoic.

TABLE 2

Effect of ETYA on the Fatty Acid Composition (mol%) of Liver Phospholipids from Trout Switched from a (n-3) Fatty Acid-rich Diet (Group I) to a (n-6) Fatty Acid-rich Diet (10- and 30-day treatment)[a]

	10 Days		30 Days	
Fatty acid	Control	ETYA	Control	ETYA
S.Sat	33.6 ± 1.8	30.8 ± 0.8	32.5 ± 1.3	31.7 ± 1.0
S(n-9)	15.6 ± 0.8	20.0 ± 4.6	15.3 ± 1.7	16.0 ± 0.8
S(n-7)	5.5 ± 1.4	7.6 ± 2.2	3.9 ± 1.0	5.2 ± 0.9
18:2(n-6)	5.8 ± 0.4	12.4 ± 2.9	6.8 ± 0.8**	11.9 ± 0.7
20:3(n-6)	2.9 ± 0.5	4.8 ± 1.0	3.2 ± 0.1**	4.7 ± 0.3
20:4(n-6)	8.2 ± 0.7	4.7 ± 1.3	10.7 ± 1.3	8.0 ± 0.7
22:5(n-6)	1.7 ± 0.4	1.7 ± 0.5	10.1 ± 0.8***	4.7 ± 0.3
S(n-6)	20.5 ± 1.3	26.3 ± 5.3	34.3 ± 2.3	32.7 ± 0.4
18:3(n-3)	0.9 ± 0.3	0.4 ± 0.03	—	—
22:6(n-3)	20.2 ± 2.1*	13.1 ± 1.4	12.7 ± 1.3	12.1 ± 1.9
S(n-3)	24.8 ± 2.9*	15.3 ± 1.6	13.9 ± 1.6	14.4 ± 2.5

[a]Values are mean ± S.E. for three preparations

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for differences induced by the drug (Student's t-test).

S, sum of the fatty acids.

Only the main fatty acids are listed.

TABLE 3

Effect of ETYA on the Fatty Acid Composition (mol%) of Liver Phospholipids from Trout Switched from a (n-6) Fatty Acid-rich Diet (Group II) to a (n-3) Fatty Acid-rich Diet (10- and 30-day treatment)[a]

	10 Days		30 Days	
Fatty acid	Control	ETYA	Control	ETYA
S.Sat	35.3 ± 1.1	39.2 ± 3.9	30.9 ± 1.3	30.5 ± 1.2
S(n-9)	15.8 ± 0.4	19.0 ± 1.8	13.4 ± 1.4	13.7 ± 0.4
S(n-7)	5.0 ± 0.4	8.7 ± 1.2	3.0 ± 0.3	2.4 ± 0.1
18:2(n-6)	3.8 ± 0.4	3.8 ± 0.1	5.7 ± 1.2	6.5 ± 0.9
22:5(n-6)	4.0 ± 1.4	2.6 ± 0.7	2.9 ± 0.3	1.8 ± 0.2
S(n-6)	18.5 ± 3.3	13.2 ± 1.3	20.5 ± 1.9	19.3 ± 2.3
18:3(n-3)	1.0 ± 0.1	1.6 ± 0.1	1.3 ± 0.4	2.7 ± 0.4
20:5(n-3)	2.9 ± 1.2	2.8 ± 0.5	2.9 ± 0.5	2.9 ± 0.2
22:6(n-3)	18.7 ± 0.4*	13.1 ± 1.5	25.6 ± 0.9	24.9 ± 1.6
S(n-3)	25.4 ± 2.3	19.9 ± 2.4	32.2 ± 0.3	34.0 ± 1.4

[a]Values are mean ± S.E. for three preparations.

*, $P < 0.05$ for differences induced by the drug (Student's t-test).

S, sum of the fatty acids.

Only the main fatty acids are listed.

content occurred in the liver phospholipids of trout from group I treated during 30 days (see Table 2), together with a 53% decrease in the content of 22:5(n-6). A 35% decrease in the 22:6(n-3) content also was observed but only after a 10-day treatment. A similar decrease in 22:6(n-3) content was the only significant modification observed in the fatty acid composition of liver phospholipids in trout fed a (n-3) rich-diet (group II, see Table 3).

Brush border membrane PE and PC of ETYA-treated trout from the group I presented an accumulation of 18:2(n-6) already detected after 10 days but an accumulation of 20:3(n-6) and a decrease in the 22:5(n-6) content were detected only after a 30-day treatment when compared with untreated fish (Table 4). The (n-3) fatty acid content remained unaltered in PE and PC.

In brush border membranes from the group II, ETYA induced a decrease in the saturated fatty acid content of PC (Table 5) together with a slight accumulation of linolenic acid; this increase occurred also in PE but only after a 10-day treatment. Since similar effects could be

TABLE 4

Effect of ETYA on the Fatty Acid Composition (mol%) of PC and PE from Intestinal Brush Border Membrane of Trout Switched from a (n-3) Fatty Acid-rich Diet (Group I) to a (n-6) Fatty Acid-rich Diet (10- and 30-day treatment)[a]

		10 Days		30 Days	
Fatty acid	Phospholipid	Control	ETYA	Control	ETYA
S.Sat	PC	40.1 ± 1.6	26.9 ± 7.0	38.7 ± 1.8	35.4 ± 0.6
	PE	37.4 ± 3.0	28.6 ± 2.5	37.9 ± 3.5	32.2 ± 1.7
S(n-9)	PC	16.8 ± 0.3	15.0 ± 1.5	15.5 ± 0.8	15.3 ± 0.2
	PE	14.0 ± 1.9	15.3 ± 1.2	13.7 ± 0.9	15.4 ± 0.9
18:2(n-6)	PC	17.7 ± 1.6**	35.4 ± 3.2	17.5 ± 1.7*	26.1 ± 1.2
	PE	10.6 ± 1.1**	23.6 ± 2.4	10.1 ± 0.6**	16.6 ± 0.7
20:3(n-6)	PC	3.9 ± 0.5	5.6 ± 1.0	4.3 ± 0.3*	5.7 ± 0.1
	PE	2.3 ± 0.6	3.7 ± 0.3	2.2 ± 0.3*	4.5 ± 0.5
20:4(n-6)	PC	5.0 ± 1.1	2.2 ± 0.5	6.9 ± 0.4	6.3 ± 0.5
	PE	6.7 ± 0.9	4.7 ± 0.5	8.2 ± 0.9	7.7 ± 0.5
22:5(n-6)	PC	3.3 ± 0.8	1.4 ± 0.4	8.9 ± 0.7**	3.3 ± 0.2
	PE	6.3 ± 1.9	3.3 ± 0.8	15.8 ± 1.8*	10.2 ± 0.6
22:6(n-3)	PC	8.3 ± 1.9	4.0 ± 0.8	3.6 ± 0.3	3.2 ± 0.5
	PE	16.4 ± 2.1	13.8 ± 2.7	6.8 ± 0.7	10.2 ± 1.0

[a]Values are mean ± S.E. for three preparations.

*, $p < 0.05$; **, $p < 0.01$ for differences induced by the drug (Student's t-test).

S, sum of the fatty acids.

Only the main fatty acids are listed.

TABLE 5

Effect of ETYA on the Fatty Acid Composition (mol%) of PC and PE from Intestinal Brush Border Membrane of Trout Switched from a (n-6) Fatty Acid-rich Diet (Group II) to a (n-3) Fatty Acid-rich Diet (10- and 30-day treatment)[a]

		10 Days		30 Days	
Fatty acid	Phospholipid	Control	ETYA	Control	ETYA
S.Sat	PC	37.9 ± 0.3**	31.0 ± 1.2	38.9 ± 0.2*	33.8 ± 1.7
	PE	36.1 ± 3.4	28.3 ± 1.1	35.2 ± 2.8	31.0 ± 2.0
S(n-9)	PC	16.2 ± 0.9	14.6 ± 0.6	19.6 ± 0.6*	16.6 ± 0.4
	PE	16.8 ± 0.2	16.5 ± 0.6	19.8 ± 1.3	18.9 ± 0.6
S(n-6)	PC	13.5 ± 0.5	14.4 ± 0.6	11.3 ± 0.4	11.4 ± 0.4
	PE	14.0 ± 1.2	15.4 ± 1.0	10.6 ± 1.4	12.7 ± 2.7
18:3(n-3)	PC	10.5 ± 0.5**	16.3 ± 0.8	8.8 ± 1.0*	13.7 ± 1.0
	PE	5.1 ± 0.6*	8.2 ± 0.3	4.1 ± 0.7	6.2 ± 1.5
20:4(n-3)	PC	1.0 ± 0.1	1.2 ± 0.1	0.8 ± 0.1	1.5 ± 0.3
	PE	0.6 ± 0.1	0.6 ± 0.1	0.3 ± 0.1*	0.5 ± 0.1
20:5(n-3)	PC	5.7 ± 0.2	6.1 ± 0.2	4.8 ± 0.5	6.2 ± 1.0
	PE	4.8 ± 0.3	5.2 ± 2.5	4.3 ± 0.2	4.8 ± 1.0
22:6(n-3)	PC	9.6 ± 0.9	10.2 ± 1.4	11.5 ± 1.5	11.0 ± 0.7
	PE	20.3 ± 2.1	22.1 ± 1.4	22.6 ± 1.7	21.1 ± 3.8

[a]Values are mean ± S.E. for three preparations. *, $p < 0.05$; **, $p < 0.01$ for differences induced by the drug (Student's t-test).

S, sum of the fatty acids.

Only the main fatty acids are listed.

observed in phosphatidylserine and phosphatidylinositol, only the fatty acid composition of PE and PC is reported in the tables.

DISCUSSION

In contrast with previous results concerning mammals (15), ETYA had no effect on the cholesterol level either in the liver or in the intestinal brush border membrane of rainbow trout.

The observed accumulation of linoleic acid induced by the drug in both liver and brush border membrane phospholipids of trout fed the (n-6)-rich diet is similar to what was reported for rat liver (16). It is likely that this accumulation is the result of a Δ6-desaturase inhibition, this enzyme system being present with similar activities in trout liver and enterocytes (unpublished results). The Δ5 desaturation step is less sensitive to the drug since a slight accumulation of 20:3(n-6) occurs only after a 30-day treatment. The important inhibition of the 22:5(n-6) synthesis induced by the drug is likely the consequence of the inhibition of the two first desaturation steps rather than that of the Δ4-desaturase since there is no accumulation of 20:4 and 22:4(n-6).

The observed decrease of the 22:6(n-3) content in trout liver after a 10-day treatment with ETYA, regardless of the diet, is unlikely the result of a biosynthesis inhibition since no accumulation of its precursors is detected. Hence, this decrease can be due either to an inhibitory effect on the deacylation-reacylation system of 22:6(n-3)-rich phospholipid species or to an activation of lipoxygenases. Although ETYA has been shown to exert inhibitory effects on trout 14-lipoxygenase (17), the lack of information about the cellular concentration of the drug in our experiments prevents further comments. After a 30-day treatment, no differences remain between treated and untreated groups, which suggests a tight regulation of the essential 22:6(n-3) liver content.

Since only one enzyme molecule almost certainly is required for the Δ6 desaturation of both (n-6) and (n-3) fatty acids (18), the strong inhibition of the trout liver Δ6-desaturase by ETYA only with 18:2(n-6) as substrate suggests that two different sites for either 18:2(n-6) or 18:3(n-3) should be present on the same enzyme molecule. In the trout intestinal epithelium, only one common site should be present since ETYA has the same inhibitory effect on both (n-6) and (n-3) substrates.

Therefore, it seems that the main target reaction of ETYA is the first desaturation step of linoleic acid in both trout liver and intestine. Still, one cannot exclude an inhibition of eicosanoids metabolism by the drug (3), which also should lead to an accumulation of 20:4(n-6) precursors. Furthermore, this work allows the demonstration of some effects of ETYA on the metabolism of (n-3) fatty acids, which are essential compounds in trout (19,20).

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Short-Term Changes in Hepatic HMG-CoA Reductase in Rats Fed Diets Containing Cholesterol or Oat Bran

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In vivo regulation of hepatic HMG-CoA reductase (HMGR) (mevalonate:NADP$^+$ oxidoreductase [acylating CoA]; EC 1.1.1.34] by phosphorylation/dephosphorylation has not been demonstrated. Rats were meal-fed semipurified diets; effects of inclusion of cholesterol (2%) or oat bran (15%) in a single meal on expressed (phosphorylated) and total (dephosphorylated) activities of HMGR were measured from 15 min to 4 hr after presentation of the meal. Expressed activity was not significantly altered in response to the control diet during the time periods examined, while total HMGR activity declined by 15 min and increased through 4 hr to an activity about 1.5 times control levels. Addition of cholesterol resulted in little change in expressed activity but a greater and more sustained reduction in total activity. Oat bran caused reductions in both total and expressed activities, which were maintained through 4 hr. Total HMGR activity was best correlated with apparent demand for cholesterol synthesis.
Lipids 22, 1057–1059 (1987).

It has been proposed that activity of the rate-limiting enzyme of cholesterol biosynthesis, β-hydroxy-β-methylglutaryl-CoA reductase (HMGR; 1), can be regulated by reversible phosphorylation of the enzyme in response to hormones (2). Ingebritsen et al. (3) have demonstrated that the degree of phosphorylation and activity of HMGR in isolated rat hepatocytes can be manipulated by addition of glucagon or insulin to the incubation medium. Evidence for operation of such a mechanism in vivo has been less consistent. Brown et al. (4) reported that modulation of rat microsomal HMGR was not changed when rats were subjected to light cycling, fasting, stress, cholesterol feeding or cholestyramine feeding. Arebalo et al. (5) found that feeding a cholesterol-containing meal to rats resulted in an inhibition of liver microsomal HMGR within 1 hr and that this inhibition could be reversed by incubation of microsomes with phosphatase.

In order to properly investigate this problem in vivo, HMGR activity must be measured within a sufficiently short time following a meal. While the study of Arebalo et al. (5) measured the activity 60 min following the meal, a mechanism dependent on hormone-stimulated phosphorylation would operate within minutes of the start of the meal, due to increased circulating insulin levels. Furthermore, no addition to the diet other than cholesterol has been tested for possible effects on HMGR activity. We report the results of an experiment that followed the course of expressed and total HMGR activity from 15 min to 4 hr following a meal. In addition, we tested the effect of a diet with added oat bran, which has been useful in lowering serum cholesterol in humans (6,7), on the time course of HMGR activity.

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Abbreviations: HMGR, HMG-CoA reductase; SP, control semipurified diet; CHL, cholesterol diet; OB, oat bran diet; R_t, total HMGR activity; R_a, expressed HMGR activity.

MATERIALS AND METHODS

Animals were housed under conditions of constant temperature and humidity, lighted from 9 p.m. to 9 a.m. Animals had access to deionized water ad libitum. Male Wistar rats (Harlan-Sprague-Dawley, Indianapolis, IN) weighing 80–116 g were fed a semipurified (SP) diet (50% glucose-hydrate, 20% casein [high nitrogen], 10% corn oil, 15% cellulose, 4% AIN mineral mix and 1% AIN vitamin mix) ad libitum. After 10 days, animals were gradually adapted to meal feeding that consisted of a daily, 2 hr meal at the onset of the dark period and were maintained on the meal pattern regimen for the duration of the experiment. On the final day of the experiment (day 28), animals were randomly assigned to one of three diet groups (18 animals per group) and fed a meal of SP diet, SP diet with 2% cholesterol (CHL; added at the expense of glucose-hydrate) or SP diet with 15% oat bran in place of cellulose (OB). Three animals from each diet group were killed at 0, 15, 30, 60, 120 and 240 min after the beginning of the meal. Liver microsomal fractions were isolated in the presence and absence of fluoride and total HMGR activity (R_t) measured in the absence of fluoride with added protein phosphatase and expressed activity (R_a) on fluoride-containing samples (8). All enzyme activity determinations were carried out in triplicate. Data were compared by analysis of variance with each diet-time group considered as a treatment (0 time and 3 diets at 5 time points yielding 16 treatments). Where appropriate, means were compared using least significant differences based on the pooled standard error from this analysis (33 d.f.; $p < 0.05$).

RESULTS

If HMGR activity is modulated by insulin levels (2), expressed activity of microsomal HMGR (R_a) would be expected to rise shortly after the beginning of the meal. Contrary to this hypothesis, R_a values in animals fed SP diet did not vary significantly during 4 hr after the start of the meal (89 vs 126 pmol/min mg, Fig. 1). By contrast, HMGR total activity (R_t) was reduced significantly, from 1063 pmol/min mg at time 0 to 372 pmol/min mg after 60 min and then increased through 4 hr (1629 pmol/min mg, Fig. 2).

Addition of cholesterol to SP diet caused a much greater reduction in R_a (Fig. 1) than that seen in SP-fed animals, and this reduction was maintained throughout the 4 hr period. Although analysis of variance did not indicate that this change was statistically significant, its magnitude (89 pmol/min mg at time 0 to 10 pmol/min mg at 30 min) and consistency suggest that it is biologically

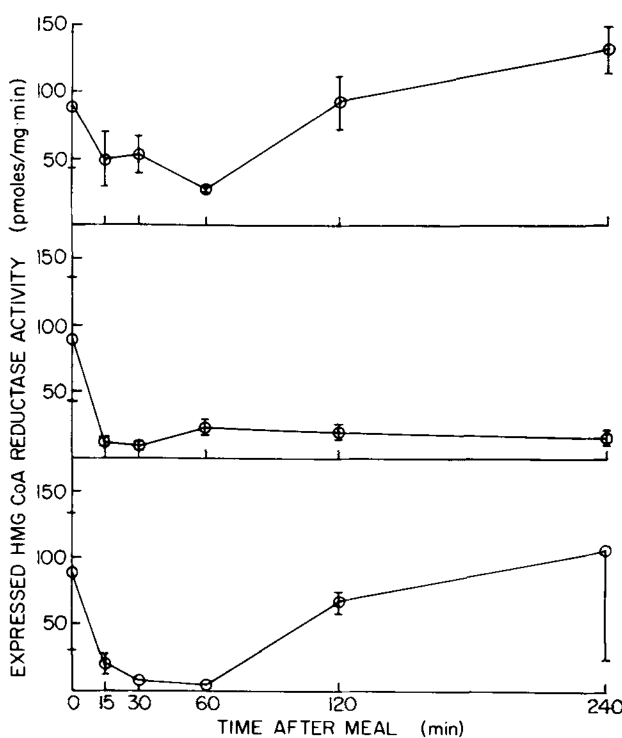

FIG. 1. Effects of dietary cholesterol or oat bran on expressed hepatic HMG-CoA reductanse activity in rats. Results from semipurified diet-fed animals are shown in the top, from cholesterol-fed in the center and from cellulose-fed in the lower figure. Rats were trained to eat meals, were presented control, oat bran or cholesterol diet at time 0 and were killed at the times indicated. Each point represents mean and standard error of activity in livers from 3 animals.

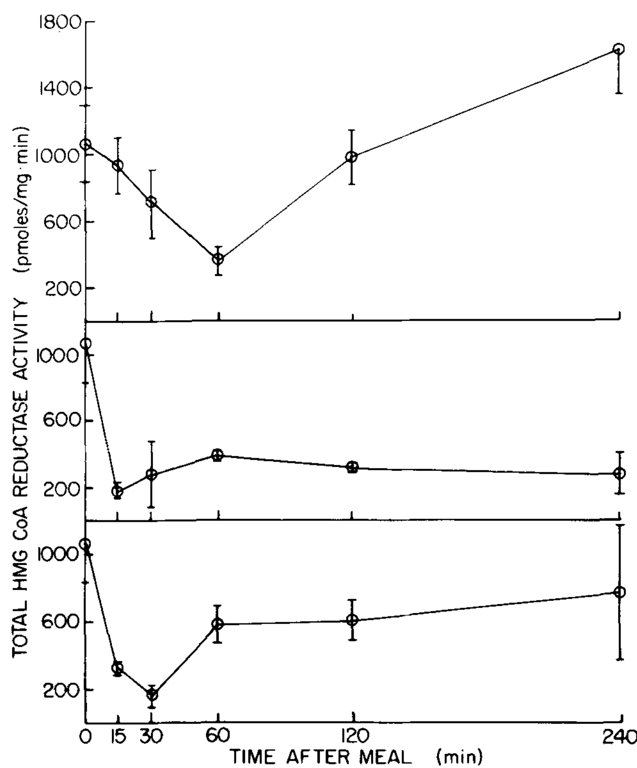

FIG. 2. Effects of dietary cholesterol or oat bran on total hepatic HMG-CoA reductase activity in rats. Results from semipurified diet-fed animals are shown in the top, from cholesterol-fed in the center and from cellulose-fed in the lower figure. Rats were trained to eat meals, were presented control, oat bran or cholesterol diet at time 0 and were killed at the times indicated. Each point represents mean and standard error of activity in livers from 3 animals.

significant. The pattern of R_t in CHL-fed animals differed from that of the SP-fed group (Fig. 2). Total enzyme activity was reduced to 182 pmol/min mg after 15 min and remained low, reaching a maximum of only 388 pmol/min mg at 60 min and remaining constant through 4 hr. Activities at all time points from 15 to 240 min were significantly lower than activity at time 0, indicating an inhibition of HMGR by dietary cholesterol.

Animals fed oat bran also exhibited reduction in R_a immediately after initiation of the meal, reaching a low of 5 pmol/min mg at 60 min (Fig. 1). Activities returned to levels similar to those present before the meal by 4 hr. Analysis of variance again indicated that none of these differences was significant. Total activity decreased to a low value of 163 pmol/min mg at 30 min, returned to 582 pmol/min mg by 60 min and continued to increase through 240 min, when it reached an activity of 776 pmol/min mg (Fig. 2). This level of activity was not significantly different from the total activity at time 0.

DISCUSSION

Several groups have endeavored to answer the question of whether HMGR is regulated by reversible phosphorylation in vivo and the additional issue of what role dietary cholesterol may play in such a regulatory scheme. Brown et al. (4) reported expressed and total activities of HMGR in rats exposed to differences in light cycling, fasting, stress and cholesterol or cholestyramine feeding. They concluded that changes in the rate of cholesterol synthesis in liver slices could not be accounted for by differences in the phosphorylation state of HMGR. Changes in R_t best reflected cholesterol synthesis rates over a wide range (50-fold) of activities. It was suggested that the large proportion of HMGR present in an inactive (phosphorylated) form in vitro was a reservoir of activity that could be called on in the short term. We observed no such change in the present studies. Our observations tended to agree with those of Brown et al. (4): that R_t was best correlated with expected cholesterol synthetic rate after a meal. These effects on R_t are consistent with the work of Dugan et al. (9), who showed a dependence of rat liver HMGR activity on the feeding status of animals.

Data presented here suggest that changes in HMGR in response to food take place in a very short period of time and that differences in diet composition can influence the magnitude of these changes. Dietary cholesterol causes a more rapid reduction in HMGR activity, which is of a greater magnitude than that produced by the SP diet alone (83% vs 65% maximum reductions). Interestingly, substitution of oat bran for cellulose causes a response similar to that of added cholesterol in a similar time frame. This could result from some absorbable component present in oat bran (10) or from some change in chylomicron size and/or composition, resulting in their more rapid clearance and uptake by the liver. These changes confer additional importance to the examination

of changes in the chylomicron-to-liver movement of lipids in response to dietary manipulation.

ACKNOWLEDGMENTS

The authors would like to thank James N. Thomas and Marilyn S. Petro for their technical assistance. This work was supported in part by the Indiana Agricultural Experiment Station (paper no. 11,206), Quaker Oats Co. and the Showalter Foundaion.

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The Filamentous Fungus *Mortierella alpina*, High in Arachidonic Acid

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Arachidonic acid is not available readily, although it is widely distributed in animal tissue. We found that in some strains of *Mortierella alpina*, arachidonic acid accounted for 68.5–78.8% of the total fatty acids. This is more than twice the arachidonic acid content of any organism previously reported. The content of arachidonic acid per dry cell weight was about 25%. Our findings offer a method for the efficient isolation of arachidonic acid in large amounts.
Lipids 22, 1060–1062 (1987).

Arachidonic acid is present widely in the animal kingdom. It has been isolated from the lipids extracted from pig adrenal gland or pig liver and from sardines, which also are an important source of eicosapentaenoic and docosahexaenoic acids. However, the arachidonic acid content is usually less than 5%, and the yield per dry weight is 0.2% or lower. Hence, large scale production of the acid is difficult.

Methods for culturing microorganisms that would be capable of producing larger amounts of arachidonic acid (1) have been proposed. Iizuka (2) reported on arachidonic acid-producing microorganisms belonging to the genera *Penicillium, Cladosporium, Mucor, Fusarium, Hormodendram, Aspergillus* and *Rhodotorura* that are cultured in media containing hydrocarbons or carbohydrates as carbon sources. This approach can produce an arachidonic acid content up to 7.5% of total lipid weight or less than 1% of dry cell weight. It also has been reported (3) that some strains of the genera *Entomophthora, Delacroixia, Conidiobolus, Pythium* and *Phytophthera* produce relatively high arachidonic acid levels. Arachidonic acid amounted to 27.1% of total fatty acid in *Entomophthora exitialis,* 19.1% in *E. ignobilis* and 18.8% in *E. thaxteriana.* It also has been shown (4) that dried mycelia of *Mortierella renispora* contain 4.8% of lipids, 26.7% of which is arachidonic acid. Ahern (5,6) showed that the red alga *Porphyridium cruentum* produces arachidonic acid with a potential yield of 1–8% of the total dry cell weight.

However, the arachidonic acid content in these species still is not sufficiently high. Hence, it was this study's aim to find microorganisms that would be capable of more efficiently producing arachidonic acid-containing lipids.

MATERIALS

The Institute for Fermentation Osaka, Japan (IFO), and American Type Culture Collection (ATCC) Rockville, MD, supplied the strains of *Mortierella alpina* used: IFO 8568, ATCC 16266, ATCC 32221, ATCC 42430.

Methyl arachidonate (purity 99%) was purchased from Nu Chek-Prep. Inc., Elysian, MN. Malt agar medium was purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Reversed-phase thin layer chromatographic (TLC) plates, RP-18F, were a product of Merck, Darmstadt, West Germany.

Gas-liquid chromatography was carried out using a Hitachi 663-50 gas chromatograph and a 1.5-m glass column of 3 mm bore packed with diethyleneglycol succinate (DEGS) 15%, SE-30 or OV-101. The instrument was fitted with a flame ionization detector. Gas chromatography-mass spectrometry was done on a Hitachi M-80 and followed by data analysis on a Hitachi M-003. ^{1}H-NMR and ^{13}C-NMR spectra were measured using a JEOL Model FX-90Q instrument.

METHODS

Ninety g of malt agar medium was added to 2,000 ml of distilled water and autoclaved for 15 min. The resulting medium was poured into 100 sterilized dishes that were 80 mm in diameter. Four strains of *M. alpina* (IFO 8568, ATCC 16266, ATCC 32221 and ATCC 42430) were inoculated each on 25 malt agar plates and incubated at 25 C for 20 days. On the 6th, 9th, 12th, 15th and 20th day after inoculation, mycelia of IFO 8568 growing on five plates were collected with a spatula and measured for growth and lipid changes. The mycelia of the other cultures similarly were collected, dried in an evacuated desiccator and then crushed by mortar and pestle using chloroform/methanol (2:1, v/v). The lipids were extracted with chloroform/methanol (2:1, v/v), and the solvent was evaporated. The lipids obtained then were converted to methyl esters with sodium methoxide. The fatty acid composition of the esters was analyzed by gas chromatography.

For the preparative purification of arachidonate, 50 mg of methyl esters that was obtained by the esterification of the total lipids from *M. alpina* IFO 8568 was subjected to reversed-phase TLC RP-18F using methanol/acetonitrile (1:1, v/v) as a developing solvent. A band at Rf 0.41 was scraped off, and the lipids were recovered to give 35 mg of methyl arachidonate with a purity of 95.9% (the remaining 4.1% being methyl γ-linolenate).

RESULTS AND DISCUSSION

Mycelium was not observed in any of the cultures until the third day after inoculation. Mycelia collected from five plates of *M. alpina* IFO 8568 from the 6th, 9th, 12th, 15th and 20th day of incubation gave the growth results, methyl esters profiles, and the increase in arachidonic acid shown in Figure 1.

The fungus growth reached a maximum at about 12 days, as did the amounts of total methyl esters and arachidonate. However, the percentage of arachidonate in the methyl esters reached a plateau three days later. When fatty acids other than arachidonic acid were produced beyond a certain level, it appears that excess acids were metabolized to arachidonic acid.

Figure 2 shows the changes in the composition of fatty acid methyl esters obtained from IFO 8568 mycelia between six and 20 days. The sum of the six methyl ester percentages at each incubation time stayed near 92.4 ± 1.5%. Arachidonic acid increased dramatically throughout the incubation whereas palmitic, stearic, oleic, linoleic and γ-linolenic acids decreased. The content of α-linolenic

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Abbreviations: DEGS, diethyleneglycol succinate; TLC, thin layer chromatography.

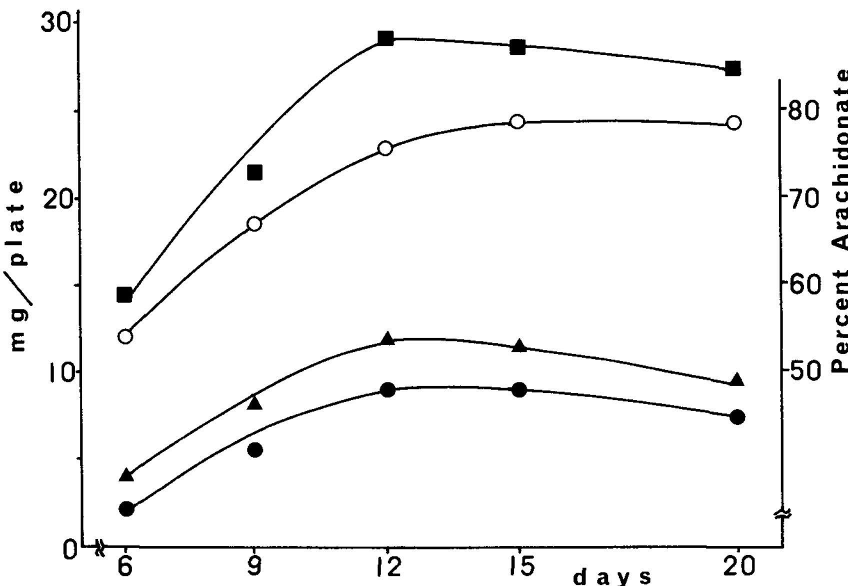

FIG. 1. Growth of *Mortierella alpina* IFO 8568 on malt agar plates at 25 C. Dry cell weight (■), weight of total methyl esters (▲), weight of methyl arachidonate (●) and content of methyl arachidonate (○).

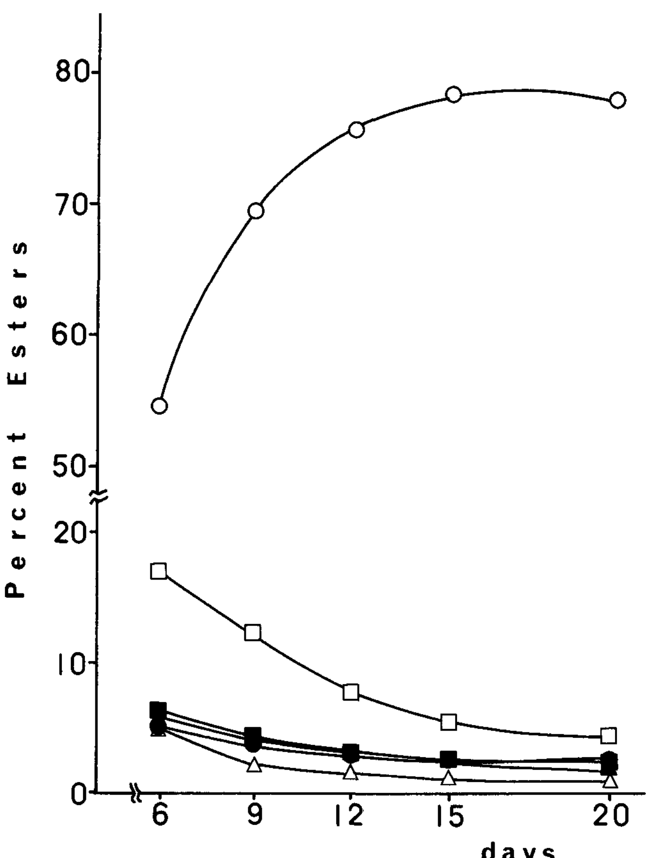

FIG. 2. Changes of methyl esters obtained from *Mortierella alpina* IFO 8568 grown on malt agar plates at 25 C. Palmitate (□), stearate (■), oleate (△), linoleate (▲), γ-linolenate (●) and arachidonate (○).

acid was less than 1%. As is shown in Figure 1, the weight of fatty acid methyl esters excluding arachidonate was constant, but the fatty acid compositions were changing markedly. This may suggest that the metabolic pathway from C16:0 → C18:0 → C18:1 → C18:2 → γ-C18:3 → C20:3(dihomo-γ-linolenic acid) → C20:4 exists also in the fungus. Dihomo-γ-linolenic acid, a precursor of arachidonic acid, amounted to only a small percentage of the total fatty acids, while the content of γ-linolenic acid was a few percent. It appears that younger cells contain higher percentages of saturated fatty acid, and older cells accumulate arachidonic acid.

Arachidonic acid content in the total fatty acids of the four strains of *Mortierella* was 68.5–78.8%, the highest value of which was 2.9 times higher than that from *E. exitialis* (3), the highest source previously known. The arachidonic acid content per dry cell weight was 22.2–26.4%, which was more than 20 times higher than the value reported by Iizuka (2) and three times higher than that reported by Ahern (5,6). The other four strains we examined gave similar values, but other *M. alpina* strains, such as ATCC 8979 and ATCC 36965, did not show exceptional arachidonic acid production when cultured on the malt agar medium.

Experiments with *M. alpina* IFO 8568 showed that similar percentages of arachidonic acid were contained in the fatty acids of neutral and polar lipid fractions, although the amount of total fatty acids from neutral lipids was about five times greater than that from polar lipids.

Methyl arachidonate was identified as all *cis* methyl eicosa-5,8,11,14-tetraenoate using the following criteria:

Elemental analysis. Found: C:79.34%, H:11.21%. Calcd: C:79.15%, H:10.77%.

Gas chromatography. Retention times of the sample on DEGS 15% (column temperature 190 C), SE-30 (column temperature 170 C) and OV-101 (column temperature 170 C) coincided with those of an authentic standard.

Gas chromatography-mass spectrometry. The mass fragmentation pattern obtained by separating the sample on DEGS 10% (column temperature 200 C) and ionizing at 70 eV corresponded closely to that of the authentic standard. The parent peak appeared at m/e 318.

^{1}H-NMR spectrometry. The ^{1}H-NMR spectrum for the sample resembled that of the standard. Taking the three methyl protons of the methyl ester group at 3.6 parts per million (ppm) as standard, there were eight protons (5.0–5.7 ppm) associated with carbon-carbon double bonds and six protons (2.6–3.3 ppm) with methylene groups between double bonds consistent with the structure of a methylene-interrupted tetraenoate. Signals derived from *trans* double bond protons were not detected.

^{13}C-NMR spectrometry. The spectra showed signals at 13.8, 22.4, 24.7, 25.5, 26.4, 27.0, 29.2, 31.4 and 33.0 ppm due to methylene carbon, at 51.2 ppm for the ester methyl carbons, and at 127.0, 127.3, 127.6, 127.9, 128.3 and 129.8 ppm for the olefinic carbons. Signals due to carbons of *trans* double bond were not detected. The spectrum was identical to that of the authentic standard.

In conclusion, we found that mycelia of *M. alpina*, which were incubated on malt agar plates at 25 C for 15 days, produced lipids, the fatty acid of which contained up to 78.8% of arachidonic acid. The arachidonic acid amounted to about 25% of the total dry cell weight. This permits easy isolation and purification of arachidonic acid.

ACKNOWLEDGMENTS

The authors thank Toshihiro Kudo for his helpful comments, Kazuhiko Suzaki for his cooperation in NMR analysis and Toshiko Tezuka for her excellent technical assistance.

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ERRATUM

In the paper "Cyclic Fatty Esters: Hydroperoxides from Autoxidation of Methyl 9-(6-Propyl-3-Cyclohexenyl)-(Z)8-Nonenoate" by R. A. Awl, E. N. Frankel, and D. Weisleder, Vol. 22, No. 10, pp. 721–730, there was an error in a figure legend.

The legend for Figure 6 should have read:

FIG. 6. Capillary gas chromatography-mass spectrometry chromatogram of hydroperoxide fraction of autoxidized cyclic ester I after hydrogenation and silylation.

Gas chromatography. Retention times of the sample on DEGS 15% (column temperature 190 C), SE-30 (column temperature 170 C) and OV-101 (column temperature 170 C) coincided with those of an authentic standard.

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FOREWORD

It is becoming a tradition that in the first week of December, clinical oncologists meet with basic researchers in Göttingen. The aim of these meetings is to continue an ongoing discussion on new aspects of tumor therapy and to stimulate interaction and cooperation. In 1985, the symposium already was devoted to ether lipids. The theme was "The Cell Membrane as Target in Tumor Therapy."[1] The organizers, C. Unger, H. Eibl and G. A. Nagel, realized that the deep interest in new antitumor drugs based on the structure of lysolecithin and its analogues should have a worldwide forum. An outcome of these discussions was the announcement of the First International Symposium on Ether Lipids in Oncology, which was held in Göttingen on December 5-7, 1986.

We were pleased that all invited speakers could attend the meeting to discuss their ideas on tumor therapy. Since Otto Westphal at the Max-Planck-Institute for Immunobiology in Freiburg was involved from the beginning in the different aspects of antitumor therapy based on the use of chemically modified lysolecithins, we were grateful that he accepted our invitation to preside over the symposium.

The organization of the congress was possible only because of the generous support by several industrial sponsors, such as the Behring-Werke, Boehringer Mannheim, Ciba Geigy, Cyanamid, Degussa Pharma, Farmitalia, Renschler, Thomae, Upjohn, and by the Bundesministerium für Arbeit und Soziales. We wish to express our gratitude for their support.

To conclude, we hope to continue this effective exchange of new ideas on the development of antitumor drugs and their clinical applications when we meet again in two or three years. We are looking forward to the Second International Symposium on Ether Lipids in Oncology in Göttingen.

Hansjörg Eibl and Clemens Unger

[1] Unger, C., Eibl, H., Nagel, G.A., eds. (1987) *Aktuelle Onkologie. 34: Die Zellmembran als Angriffspunkt der Tumortherapie,* W. Zuckschwerdt Verlag, München, Bern, Wien, San Francisco.

EDITORIAL

Ether lipids have not always enjoyed regal status. Discovered more than half a century ago, ether lipids seemed important to primitive starfish and primeval sharks. At best, they looked like relics from past millenia trying to remind us that evolution had alternatives to the fatty ester bond.

How things have changed. With the discovery of platelet activating factor (PAF), we have accepted the fact that simple derivatives of ether lysophospholipids can carry cellular messages to specific receptor sites. Through the pioneering work of Otto Westphal and his colleagues, we have learned to understand that alkyl lysophospholipids (ALP) function as immunomodulators and that they deserve recognition of their own because of their rather selective antineoplastic cytotoxicity. One of the intriguing and promising aspects of tumor chemotherapy with alkyl lysophospholipids is that, unlike essentially all other antitumor agents, ALP appear to be targeted at the cell membrane rather than the nucleus. This opens up entirely new approaches to tumor therapy.

Last December, a distinguished, albeit small, group of lipid biochemists, pharmacologists and clinicians convened in the quaint university town of Göttingen to assess the state-of-the-art in ether lipid biochemistry and to tally the successes and promises of the first clinical trials with ALP as antitumor agents. The meeting was prepared meticulously by the local organizing committee under the auspices of the German Cancer Society, the Association of Medical Oncology, the Association of Experimental Oncology and the International Union Against Cancer. By all measures, the meeting was a splendid success.

This special issue of *Lipids* contains 35 peer-reviewed contributions based on papers and posters presented at this First International Symposium on Ether Lipids in Oncology. The issue is a timely document of the current knowledge in this exciting field. We at *Lipids* are proud to be part of this.

We wish to express our appreciation to Professor Otto Westphal, president of the symposium, and to Drs. Hansjörg Eibl and Clemens Unger and the other members of the organizing committee for their commitment and support. I also wish to thank the colleagues who have coedited this issue. We are indebted to the many reviewers for their effort and advice.

Finally, I would like to express my sincere gratitude to Professor Helmut K. Mangold. He not only was instrumental in bringing about this issue but he introduced me to the field of ether lipids when I joined his laboratory as a postdoctoral fellow just 25 years ago. He never left any doubt in my mind that ether lipids deserve regal status.

Wolfgang J. Baumann
Editor